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Toxoplasmosis

Pathology, Histopathlogy and Serology

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1. Introduction

Toxoplasmosis is a protozoan disease recorded from a very wide range of vertebrate species (placental and marsupial mammals, birds and reptiles). The parasite is distributed throughout the world with the greatest prevalence of infection in temperate climates, especially in shaded, moist habitats which favour the survival of oocysts.

The definitive hosts are domestic and wild felids; however, *Felis catus* is undoubtedly the single most important source of infection for humans and domesticated livestock. Sexual replication occurs in the cat's small intestine with unsporulated *oocysts* being passed in their faeces. After five to seven days, the sporulated oocysts are infective to intermediate hosts. Herbivorous animals normally acquire infection from oocysts contaminating their plant foodstuffs. After ingestion the oocysts release the *sporozoite* stage which pass across the gut and enter the body via the lymphatic or vascular system. The so-called *trophozoite* stage can enter and divide within a large range of cell types such as neurons and other nervous tissue cells, vascular endothelium, hepatocytes, myocardial cells, skeletal muscle cells and alveolar epithelium. Within host cells the organism multiplies to form *cysts*. These cyst stages are infective not only to cats (to perpetuate the infection) but also for other meat-eating animals.

In urban and agricultural environments, introduced rodents are probably the main non-clinical intermediate hosts of *Toxoplasma gondii* thus maintaining a domestic cat–rodent cycle. Sheep are readily infected with *Toxoplasma* and mutton can be the source of infection for meat-eating animals, including humans. In addition, some coprophagic and soil-borne insects can act as transport hosts for *Toxoplasma* oocysts. Consequently, insectivorous animals are also prone to infection.

The clinical manifestation of toxoplasmosis can, therefore, be variable depending on whether it is manifesting as an multiple organ/tissue disease or causing discrete clinical signs referable to a specific organ system (e.g. nervous, respiratory, cardiac, lympho-reticular). In certain environments which favour the survival of *Toxoplasma* oocysts and among certain people (cat owners, abattoir workers, veterinarians, young children, raw or partially cooked meat-eaters) exposure and infection is more likely. Since there are several ways that animals and humans can acquire infection (food and fomite contamination with oocysts, tissue cysts, and through insect contamination), the presence of a serological reaction to this parasite may not be unexpected. Intracellular *bradyzoites* can remain viable for long periods (year or decades), so inapparent (latent) infection is not uncommon in many species.

Expression of clinical disease usually occurs in naive animals exposed to large numbers of infective organisms or as a result of a lowering of the host's immunocompetence through a range of stressor factors (nutritional, psychological, hormonal or environmental), debilitation due to concomitant infections [e.g.human immunodeficiency virus (HIV) and senility or chemotherapy (cortico-steroids or cytotoxic agents]. These circumstances may increase susceptibility to infection and disease expression or it may reactivate latent infection.

Infection of some species of mammals during pregnancy can result in transfer of the organism into the placenta and foetus, resulting in foetal deaths, abortions, stillbirths or developmental abnormalities (particularly affecting the nervous system).

Toxoplasma gondii is highly immunogenic, stimulating protective cell-mediated immune responses but less protective humoral antibody responses. The prevalence of antibodies to *Toxoplasma* is generally much higher than the incidence of clinical disease as exposure to infection in healthy animals is well tolerated leading to carrier status and resistance to disease following reexposure.

Marsupials, ring tailed lemurs (*Lemur catla*) and New World primates appear to be very susceptible to infection. Among domesticated animals, toxoplasmosis is one of the main infectious causes of reproductive wastage (barren females, abortions, stillbirths, weak lambs) in goats and sheep.

The consequences of infection in an individual are influenced by the pathogenicity of the *Toxoplasma*, the infecting dose and the parasite stage ingested, the age, sex and immunocompetence of the host and the susceptibility of the host species. Interpretation of *Toxoplasma* serological reactions needs to take these factors as well as the nature of the clinical history into consideration. It is important to select the appropriate diagnostic procedures which assist in differentiating between clinical toxoplasmosis and seroconversion.

2. Detection of *Toxoplasma*

Toxoplasmosis may be diagnosed by the detection and identification of certain life cycle stages of the parasite in histological sections of biopsy or autopsy material. However, finding the organism and identifying it are very difficult and require an experienced eye. The major difficulties encountered with this form of diagnosis are the very small size of the individual organisms, confusion with other sporozoan parasites, and the relative non-specificity of any associated pathology.

When it is necessary to make a diagnosis on biopsy material, specific fluorescent-antibody staining can be performed on frozen sections of unfixed tissues. While this procedure is very

sensitive, it is extremely critical and requires great expertise. Therefore, it is best supplemented by the slower procedure of staining paraffin-embedded sections of fixed tissues. These standard techniques are also employed on suitable autopsy material to substantiate diagnosis made via other techniques.

2.1. Standard Techniques

Tissues taken at biopsy or autopsy are fixed with 10% formol-saline as soon as possible after sampling, but other suitable fixatives will also suffice (e.g. glutaraldehyde, Serra's fluid). Routine reagents are used for the dehydration and embedding of the tissues and for the staining of the sections with haematoxylin and eosin (H and E) or periodic acid Schiff (PAS). Routine methods for embedding, sectioning and staining are used. Best resolution is obtained using 5–6 µm sections.

For definite diagnosis, it is essential to find and identify the organism. The life cycle stages which are detected are tissue cysts and individual or group tachyzoites. The tissue cysts are most readily detected, particularly in the central nervous system, and are fairly characteristic. They are spherical, thin-walled, variable in size (25–100 µm in diameter), and are filled with round to crescent shaped zoites (3–6 µm in length and 2–3 µm in width) which are basophilic staining and strongly PAS positive.

There may be some confusion with similar stages of other parasites, especially *Sarcocystis* spp., *Besnoitia* spp., *Hammondia* spp., *Frenkelia* spp., and *Encephalitozoon* (*Nosema cuniculi*), but this is usually overcome with experience. Some 'cysts' of *Sarcocystis* and *Besnoitia* are larger, have thicker cyst walls and larger zoites. However, some species of *Sarcocystis* and *Hammondia* have thin walls and small zoites and may be very difficult to differentiate from *T. gondii* in muscle. *Encephalitozoon* cysts are difficult to detect in H and E sections, having no defined cyst wall. They are best detected by Gram stain as the spores are Gram positive. *Frenkelia* cysts occur in the brain and differ from *Toxoplasma* by being subspherical or lobate.

Intracellular tachyzoites, singly or grouped, are very difficult to detect and are virtually impossible to identify positively as those of *Toxoplasma*. They are usually crescent shaped, measure 4–8 µm in length and 2–4 µm in width, and are found in a wide variety of tissues and organs throughout the body.

Lesions in organs tend to be necrotic, especially if acute, and are sometimes surrounded by lymphocytes, monocytes and plasma cells. However, the lesions are not sufficiently characteristic to allow definite diagnosis without positive identification of the organism itself. In aborted lambs and kids, the presence of areas of non-reactive leucomalacia and microglial nodules in the brain is highly suggestive of toxoplasmosis. In the newborn of other species and in older domestic animals, toxoplasmosis is suggested by encephalitis characterised by microglial aggregations and often associated with some degree of perivascular cuffing. It is usual for a number of organs to be involved in *Toxoplasma* infections, and lesions are frequently seen in the lungs, heart and adrenals. The lung lesions are typically an interstitial pneumonia, which may be confused with a viral pneumonitis unless the parasites are found. Non-suppurative myocarditis may be detected in the heart, while adrenal lesions are usually focal areas of necrosis. Chorioretinitis may be seen in intraocular infections and is characterised by oedema and necrosis of the retina, necrosis and disruption of the pigmented layer and the layer of rods and cones, and infiltration of the retina and choroid with inflammatory cells. Some consider this to be a hypersensitivity response, similar to that occurring with other chronic infections such as tuberculosis and syphilis.

2.2. Specialised Techniques

Tissues taken at a biopsy or autopsy are trimmed into 1 cm^3 pieces, wrapped in aluminium foil and immediately snap-frozen by immersion in either liquid nitrogen (N_2) or a 1:1 mixture of ethanol (C_2H_5OH) and dry ice (CO_2). Snap-freezing has proven to be essential to minimise loss of specificity of staining; therefore, this technique may not be feasible in all clinical situations. After snap-freezing, the tissues may be stored at -20°C until they are processed.

For direct staining, it is necessary to have suitable anti-*Toxoplasma* sera conjugated to fluorescein isothiocyanate (FITC). This serum does not have to be species-specific; therefore, it is best to standardise the source animals for each laboratory (i.e. sera from sheep experimentally infected with large doses of a strain of *Toxoplasma* of low virulence). Standard conjugation methods are used and each new batch of conjugated sera must be adequately tested for potency and specificity. It may also be purchased commercially.

For indirect staining, it is necessary to have anti-*Toxoplasma* sera and conjugated antispecies sera. As for the direct staining method, the anti-*Toxoplasma* sera do not have to be species-specific and may be obtained in the same way as above (i.e. sheep sera). However, the antispecies serum must be specific for the source animal species supplying the anti-*Toxoplasma* sera (in this case, antisheep sera raised in rabbits). This is conjugated to FITC using standard procedures and it is also tested for potency and specificity. (*N.B.* The conjugated sera are best stored in aliquots at -20°C, as repeated thawing and freezing are to be avoided. They may still be suitable for use after six months of storage but adequate controls should be employed to test them after this time.)

The frozen tissues are best processed within 30 days of sampling and are sectioned at 5–8 µm on a cryostat. The sections are air-dried for at least 20 min and then fixed in 10% methanol (CH_2OH) for five minutes at room temperature.

The prepared sections can then be stained using either the direct or indirect staining method.

2.2.1. Direct Fluorescent Antibody Staining

Drops (5 µL) of the conjugated anti-*Toxoplasma* serum are applied to the sections and they are incubated for 30 min in a moist chamber at 37°C. They are then washed for 30 min with three changes of phosphate buffered saline (PBS) (pH 7.2), and then quickly flushed with distilled water to remove any precipitated salts. The sections are then counterstained in a 1% aqueous solution of Evans Blue, mounted in phosphate buffered glycerol (pH 9.0) and are ready for examination.

2.2.2. Indirect Fluorescent Antibody Staining

The sections are covered with the anti-*Toxoplasma* sera and are incubated for 30 min in a moist chamber at 37°C after which they are washed for 30 min in three changes of PBS. They are then overlaid with the conjugated anti-species sera and incubated for a further 30 min in the moist chamber at 37°C. They are then washed for 30 min in three changes of PBS, flushed with distilled water, counter-stained, mounted in phosphate buffered glycerol and are ready for examination. (*N.B.* It is important that the sections not be allowed to dry out at any stage during their preparation.)

The preparations are best viewed immediately and prolonged exposure to light should be avoided as this decreases the strength of fluorescence. The sections are screened at x40–250 magnification and then positive identification made at x400 magnification under a microscope using alternately transmitted dark-field illumination (tungsten lamp) and incident ultraviolet (UV) illumination (mercury-vapour or iodine-quartz lamp) with an adequate light filtration system.

Fluorescent antibody staining allows for the detection and identification of the *Toxoplasma* antigen within the tissues, which is characterised by yellow–green fluorescence of the zoite's walls. Frequently, non-specific background staining makes this method of reduced value and it is only with some experience in examination and methodology (including adequate reagent controls) that this may be alleviated.

3. Isolation of *Toxoplasma*

The most convincing diagnosis of toxoplasmosis is made by the isolation of the parasite from biopsy or autopsy material via laboratory culture. Its main advantages are that a greater volume of material may be examined and that it enables the parasite to proliferate, thereby resulting in an increased level of detection. However, the time delay before diagnosis is made may be substantial; therefore rapid diagnosis, especially necessary in acute cases, is not possible. Thus, its main functions are for the diagnosis of toxoplasmosis in autopsy specimens (where the time delay is less critical) and for confirmational diagnosis in biopsy specimens.

The organism may be cultured in laboratory animals, embryonated eggs and tissue cultures. White mice are the most useful animals for culture for they are highly susceptible and rarely suffer from spontaneous infection. The culture of the organism in embryonated eggs is less sensitive than in mice, whereas tissue cultures are the least sensitive and seem to be suitable for virulent strains only. The latter two methods are used very rarely and will not be considered here.

3.1. Mouse Inoculation

Any body tissue or fluid may be examined for toxoplasmosis, and the treatment of these specimens depends upon their physical characteristics and degree of bacterial contamination.

Tissues and organs are finely ground with sterile sand and sterile 0.8% saline (containing 100 units of penicillin and 10 µg streptomycin/mL) in a glass tissue grinder and made up to 10–20% emulsion with the same diluent. Fibrous organs and muscle are best digested by a pepsin–hydrochloric acid (HCl) solution the deposit of which is mixed with sterile saline with antibiotics prior to culture. Body fluids are mixed with the same volume of sterile saline with antibiotics. All prepared samples are left to stand for one hour prior to inoculation. Each prepared sample is injected in 1 mL doses intraperitoneally into two to three mice. Alternatively, 0.05 mL doses may be injected intracerebrally, a much more sensitive route.

Mice infected with virulent strains of *Toxoplasma* usually die within 2–14 days after inoculation, and the organism may be demonstrated in peritoneal exudate or smears of the brain, liver, lung or spleen. Tachyzoites can be readily detected in peritoneal exudate and assume their characteristic appearance (crescent shaped and measuring 4–8 µm in length and 2–4 µm in width). Smears from solid organs are best stained with Giemsa to aid detection of the zoites.

Avirulent strains usually do not kill mice; therefore, they are examined by post mortem at 6–8 weeks after inoculation. Where diagnosis is uncertain, it is sometimes desirable to carry out blind passages of material (preferably the brain) from the first set of mice to a second or further set of mice following the methods outlined above. Squash preparations of the brain are the most convenient means of detecting chronic infections, and characteristic tissue cysts are

readily detected upon examination by light microscopy at x20–40 magnification. For the most accurate results, blood should also be collected from each mouse for serological testing for antibodies against *Toxoplasma*.

4. Detection of Antibodies to Toxoplasma

The diagnosis of toxoplasmosis is seldom made by the detection or recovery of the organism, as suitable specimens for histology or culture are often not available. Thus recourse is made to the detection of antibodies to *Toxoplasma* by various serological procedures. These procedures are best employed in screening programs and also as adjuncts to the diagnosis of acute toxoplasmosis. Their main advantages are that samples are readily obtainable, and that by sequential testing an indication of the status of infection may be gained and the course of infection followed. In the case of aborted or newborn lambs or kids, which have not suckled, the presence of *Toxoplasma* antibodies in blood or transudates is confirmatory of congenital infection.

Many serological tests have been applied to toxoplasmosis but only a few are used as other than research procedures. At present, the most widely used of the established techniques are the Indirect fluorescent antibody test (IFAT), the Indirect haemagglutination test (IHAT), and Enzyme-linked immunosorbent assay (ELISA) and Direct and modified agglutination test (DAT/MAT).

The IFAT and IHAT can utilise commercially available freeze-dried antigens which are adequately controlled and standardised and present no hazards in the laboratory. The ELISA is sensitive, specific, and relatively easy to perform once established, and will probably become the test of choice in a few years.

IFAT and ELISA antibodies appear early in infection and may rise to high levels, falling slightly thereafter but persisting at an elevated level for many months before declining to low levels after many years. IHAT antibodies appear slightly later than those detected by the other tests and they persist at elevate levels for many years.

4.1. The Indirect Fluorescent Antibody Test (IFAT)

This technique is based on the labelling of an antibody–antigen complex with an immunoglobulin coupled to a fluorescent marker. The immunoglobulin must be species-specific against the test sera to facilitate labelling which is directly observable as fluorescence of the antigen when viewed under UV illumination.

4.1.1. Antigen

Toxoplasma tachyzoites are passaged at three- to four-day intervals in the peritoneal cavity of mice. The exudate is diluted about 10 times in 1% formol–saline and the suspension is drawn in and out of a fine bore needle (e.g. 26 gauge) to disperse the clumps of organisms and thoroughly mix the suspension. This is allowed to stand at room temperature for 30–60 min and is then centrifuged for five minutes at 30 *g* to remove host cells and clumped parasites. The individual organisms remaining in the supernatant are packed by centrifugation for five minutes at 400 *g*, resuspended in saline and then centrifuged again for five minutes at 400 *g*. The tachyzoites are then diluted in saline to give about 20 organisms per high power field (x400). Drops of this antigen are then placed on pre-ruled, clean glass slides using a 26-gauge needle or a 3 mm bacterial loop.

The slides are air dried, fixed in methanol for 10 min and may then be stored at -20°C for up to three months prior to use.

Alternatively, the antigen may be obtained commercially in a freeze-dried state, reconstituted in the laboratory and placed on glass slides as above.

4.1.2. Conjugated Immunoglobulins

For the IFAT, it is necessary to have conjugated antispecies immunoglobulins which are specific for the various test-animal species. Antisera may be raised in the laboratory in rabbits, and the immunoglobulins may be separated from the sera by salt fractionation or ultracentrifugation. These are conjugated to fluorescein isothiocyanate using standard conjugation and purification procedures. The conjugated immunoglobulins must then be tested for potency and specificity before use. They are best stored in concentrated aliquots at -20°C and may keep for up to six months.

Alternatively, conjugated immunoglobulins may be obtained commercially.

4.1.3. Phosphate Buffered Saline

PBS (pH 7.2) is used throughout the IFAT and is prepared as follows.

NaCl	38.25 g
Disodium hydrogen phosphate, Na_2HPO_4	3.62 g
Potassium dihydrogen phosphate, KH_2PO_4	1.05 g
Distilled water to	5 L

4.1.4. Phosphate Buffered Glycerol

Prior to examination, the slides are mounted in phosphate buffered glycerol (pH 9.0) which is prepared as follows.

Sodium bicarbonate, $NaHCO_3$	0.0729 g
Sodium carbonate, Na_2CO_3	0.016 g
Distilled water to	10 mL
Glycerol to	100 mL

4.1.5. Test Procedure

The test sera are diluted in PBS in doubling dilutions (usually 1/16, 1/32, ..., 1/512) and one drop of each dilution is placed on the appropriate test area of antigen. The slides are incubated in a moist chamber at 37°C for 30 min after which they are washed for 20 min in two changes of PBS. Each antigen area is then overlaid with the appropriate conjugated immunoglobulin and the slides are incubated for a further 30 min in the moist chamber at 37°C. The slides are washed again for 20 min in two changes to PBS and then quickly flushed with distilled water to remove any precipitated salts. The slides are then mounted in phosphate-buffered glycerol and are ready for examination. *N.B.* Counterstaining with 0.5% Evans Blue prior to mounting may improve definition in some circumstances. It is important that the slides are not allowed to dry out at any stage during their preparation).

4.1.6. Controls

A negative control is run on each slide and consists of one antigen drop being treated with PBS instead of test sera. This control checks for auto-fluorescence and also determines the degree of non-specific background fluorescence. A positive control consisting of a serum of known IFAT titre is also run to test the validity of the test.

4.1.7. Examination

The preparations are positioned under a microscope at x100 or x400 magnification using transmitted dark-field illumination (tungsten lamp source). The test is then read using either transmitted or incident UV illumination (mercury-vapour or iodine quartz lamp source) with a suitable light filtration system.

A positive result is obtained when the *Toxoplasma* tachyzoites exhibit yellow–green fluorescence of the whole zoite wall. With experience, it is possible to grade the degree of fluorescence on a +++, ++, + scale. Fluorescence of only part of the wall is not regarded as significant. The endpoint of the IFAT is given as the highest titre in which 50% of the tachyzoites show complete '+' fluorescence.

With most species, a titre of 1/16 is usually regarded as indicative of infection. This criterion holds for humans and most domestic and laboratory animals, but the position with other exotic and native species has yet to be definitely established. If possible, each laboratory should endeavour to check the significant titre of each species within their own confines, for considerable variation is possible between laboratories, their criteria of significance and their batches of reagents.

4.2. The Indirect Haemagglutination Antibody Test

The IHAT technique involves the agglutination of red blood cells coated with *Toxoplasma* antigen by dilutions of antibody. The IHAT has the advantage over the IFAT that it can be used to measure *Toxoplasma* antibody in the serum of any species without having to change reagents. Also, it is easy and quick to use and is therefore ideal as a rapid screening test for large numbers of sera.

Good, reliable IHAT kits are available commercially but are expensive. The sensitised red blood cells (RBC) described here are easy and inexpensive to prepare and may be stored for up to three months at 4°C.

4.2.1. Antigen

Toxoplasma tachyzoites are passaged in mice and purified from the peritoneal exudate cells as described for the IFAT. The purified parasites (about 10^9/mL) are then lysed, ideally by ultrasonication (50 W, 20 KHz for five minutes), although repeated freezing and thawing will suffice.

4.2.2. Diluents

4.2.2.1. Buffered saline (pH 6.0)

NaCl	5.67 g
Na_2HPO_4	0.77 g
KH_2PO_4	5.33 g
Distilled water	1 L

4.2.2.2. Buffered saline (pH 8.0)

NaCl	5.67 g
Na_2HPO_4	6.01 g
KH_2PO_4	0.32 g
Distilled water	1 L

4.2.3. Preparation of Sheep Red Blood Cells

Wash RBC three times in normal saline and make up to 50% in saline. Add 4.0 mL of pyruvic aldehyde (CH_3COCHO) to 12.0 mL of 1.7% (0.3 mol/L) sodium chloride solution and adjust the pH to 7 by the addition of 1% (0.1 mol/L) sodium carbonate solution (about 35 mL). To this solution add 7.0 mL of pH 8.0 buffered saline and add 10.0 mL of the 50% RBC suspension. Leave this mixture at 4°C for 24 hours with occasional shaking. Wash the cells three times in normal saline and make up a 10% suspension in saline containing 0.1% (0.15 mol/L) sodium azide (NaN_3).

4.2.4. Sensitisation of Red Blood Cells

Prepare a 1.4% suspension of pyruvic aldehyde treated cells in pH 6.0 buffered saline. Dilute the *Toxoplasma* antigen to optimal concentration (usually about 2 mg/mL) in pH 6.0 buffered saline, and mix with an equal volume of 1.4% RBC. Incubate this mixture at 56°C for one hour, wash the cells once in saline and resuspend to a 0.7% concentration in PBS pH 7.2. Store at 5°C.

4.2.5. Test Procedure

The sera to be tested are serially diluted from 1/8 to 1/2048 in microtitre wells containing 25 µL of PBS pH 7.2. Sensitised cells (25 µL) are

added to each well from the 1/16 dilution and then left at room temperature for two hours. The last dilution showing clear formation of a carpet of cells on the bottom of the wells is taken as the endpoint. A control well containing 25 µL of a 1/8 dilution of serum and 25 µL of unsensitised 0.7% (0.1 mol/L) pyruvic aldehyde treated cells should show a clear-cut button. It is also wise to include known non-reactive and reactive sera as controls in the system.

An IHAT of 1/64 for human serum is indicative of past exposure to *Toxoplasma*.

4.3. The Enzyme-Linked Immunosorbent Assay

The ELISA is rapidly gaining popularity because large numbers of specimens can be done at once, the test can be performed in a relatively short period, and the results obtained are quantitative.

4.3.1. Antigen

Toxoplasma antigen identical to that used for the IHAT is centrifuged (3000 *g*/five minutes), and the supernatant fluid is used to coat wells of a microtitre tray.

4.3.2. Diluents

4.3.2.1. Carbonate–bicarbonate buffer pH 9.8 (0.1 mol/L)

The buffer is prepared as follows. Mix 40 mL of 0.1 mol/L sodium carbonate and 60 mL of 0.1 mol/L sodium bicarbonate.

4.3.2.2. Phosphate-buffered saline pH 7.2

See 4.1.3. and add 0.5 mL Tween 20/L (0.05%).

4.3.2.3. Para-nitrophenol phosphate

This is best purchased in tablet form ready to use from Sigma Chemical Company. Dilute one table (5 mg) in 5 mL of diethanolamine buffer.

4.3.2.4. Diethanolamine buffer

Diethanolamine, $(HOCH_2CH_2)_2NH$	97 mL
Distilled water	800 mL
NaN_3	0.2 g
Magnesium chloride, $MgSO_4.6H_2O$	0.1 g

Hydrochloric acid is added to bring the pH to 9.8 then distilled water to make up to 1 L. Store in the dark at 4°C.

4.3.3. Test Procedure

Microtitre trays are sensitised with the stock antigen preparation diluted in 0.1 mol/L carbonate–bicarbonate buffer at a protein concentration of 2.5 µg/mL. Plates are then incubated (18 hours, 4°C) and then washed with PBS containing 0.5% Tween 20 to remove excess antigen.

The plate is then precoated with 1.0% bovine serum albumin (BSA) in PBS-Tween 20 (PBST) for one hour at 37°C. After washing, test sera appropriately diluted (usually about 1/200) in PBST containing 1.0% BSA are added to wells in the plate and incubated for one hour at 37°C. Plates are washed again. Alkaline phosphatase conjugated antispecies antibody, obtained commercially and diluted to the recommended dilution, is added to all wells and incubated at 37°C for one hour. After washing, 0.1% para-nitrophenol phosphate is added, the plate is incubated for 45 min at 22°C, and the reaction is stopped by the addition of 2 mol/L sodium hydroxide (NaOH), and the Abs_{410} is measured.

Volumes (usually 100 µL) and concentrations of the reagents can be adjusted to give optimal conditions for each particular species to be tested. Approximate results to aim for are in Table 1.

Table 1. Interpretation of enzyme-linked immunosorbent assay

Absorbency at 410 nm	*Interpretation*
< 0.2	No antibody present.
0.21–0.7	Moderate amount antibody present.
> 0.71	Large amount antibody present.

4.4. Direct and Modified Agglutination Test

The DAT/MAT is another test which is being increasingly used. The test is similar to haem-agglutination test, in this case, sera are titrated and incubated with *Toxoplasma* tachyzoites in microtitre plates. The advantages of this test are:

(a) its applicability to a wide range of host species; and

(b) its ability to diagnose acute infections. Sera are mixed with 2-mercaptoethanol (SC_2H_6O) to denature the IgM immunoglobulin, a difference of two or more titration dilutions is considered suggestive of IgM-predominant seroconversion.

4.4.1. Antigen

Toxoplasma tachyzoites derived from mouse ascitic fluid suspended in formalin. (Available from BioMerieux Laboratories).

5. Acknowledgment

With thanks to D. Obendorf, Department of Primary Industry, Tasmania, who wrote the Introduction to this chapter.

6. Reference

Desmonts, G., and Remington, J.S. (1980). Direct agglutination test for the diagnosis of *Toxoplasma* infection: Method of increasing sensitivity and specificity. *Journal of Clinical Microbiology* **11**, 562–8.