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TOXOPLASMOSIS

by P.J. O'Donoghue

in collaboration with W.J. Hartley and B.L. Munday

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TOXOPLASMOSIS

Standard Diagnostic Techniques

Toxoplasma gondii is a sporozoan parasite of world-wide distribution and occurs in a wide variety of vertebrate hosts, including man. The resultant condition is known as toxoplasmosis and may be acute or chronic in nature, and the organism itself exhibits varying degrees of virulence. The diagnosis of toxoplasmosis on clinical grounds is usually difficult, thus recourse must be made to the demonstration of either the organism or antibodies against it.

CONTENTS

I. DETECTION OF TOXOPLASMA

Toxoplasmosis may be diagnosed by the detection and identification of certain life cycle stages of the actual parasite in histological sections of biopsy or autopsy material. However, finding the organism and identifying it is very difficult and requires 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 nonspecificity of any associated pathology.

HISTOLOGY

When rapid diagnosis is required for biopsy material, specific fluorescent-antibody staining may be performed on frozen sections of unfixed tissues. While this procedure is very sensitive, it is also extremely critical and requires great expertise. Therefore it is best supplemented by the relatively longer but more usual staining of paraffin-embedded sections of fixed tissues. These standard techniques are also best employed wherever possible on all suitable autopsy material to substantiate diagnosis made via other techniques.

(a) Standard techniques

(i) Materials

Tissues taken at biopsy or autopsy are usually fixed with 10% formol-saline as soon as possible after sampling, but other suitable fixatives will also suffice (i.e. glutaraldehyde, Serra's fluid, etc.).

Routine reagents are used for the dehydration and embedding of the tissues, and also for the staining of the sections with haematoxylin and eosin (H & E) and periodic acid Schiff (PAS).

(ii) Methods

Routine methods for embedding, sectioning and staining are used. Best resolution is obtained by using 5-6μ sections.

(iii) Diagnosis

For definite diagnosis it is essential to find and identify the organism. The life cycle stages which are detected are tissue cysts, terminal colonies and individual or grouped trophozoites.

The tissue cysts and terminal colonies are those most readily detected, particularly in the central nervous system, and are fairly characteristic. They are spherical, thin-walled, variable in size (5-100μ in diameter), and are filled with round to crescent shaped zoites (3-6μ in length and 2-3μ in width) which are basophilic staining and strongly PAS positive.

There may be some confusion with similar stages of other parasites, notably Sarocystis sp., Besnoitia sp. and Encephalitozoon (Nosema cuniculi), but this is usually overcome with experience. The 'cysts' of Sarcocystis and Besnoitia are usually larger, have thicker cyst-walls and comparatively larger zoites; whereas those of Encephalitozoon are similar in size but exhibit no defined cyst-wall, are less basophilic and only slightly PAS positive.

Intracellular trophozoites, 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 4-8μ in length and 2-4μ in width, and are found in a wide variety of tissues and organs throughout the body.

Lesions in organs tend to be necrotic in nature, especially if acute, and are sometimes surrounded by lymphocytes, monocytes and plasma cells. However, the lesions are not sufficiently characteristic to allow diagnosis without positive identification of the organism itself. The central nervous system may exhibit some degree of meningoencephalomyelitis characterized by necrosis, inflammatory lesions, calcification and cyst formation. The areas predominantly involved are the cortex, subcortical white matter, midbrain, pons and medulla. In aborted lambs and calves the presence of areas of non-reactive leucomalacia and microglial nodules in the brain are highly suggestive of toxoplasmosis. In the newborn of other species and in older domestic animals, the picture suggestive of toxoplasmosis is encephalitis characterized by microglial aggregations and often associated with some degree of perivascular cuffing. Also, minute scattered necrotic areas or granulomas may be seen in the brain, which may later tend to calcify. Chorioretinitis may be seen in intraocular infections and is characterized 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).

(b) Specialized techniques

(i) Materials

Tissues taken at biopsy or autopsy are trimmed into small 1 cm cubes, wrapped in aluminium foil and immediately snap-frozen by immersion in either liquid nitrogen or a 1:1 mixture of ethanol and dry ice. Snap-freezing has proven to be essential to minimize 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.

The reagents for fluorescent-antibody staining depend upon the staining method to be employed. (For details refer to D.M. Weir (ed.) "Handbook of Experimental Immunology". Vol. 1-3, 2nd. ed. 1973, Blackwell Scientific Publications).

Direct staining:

It is necessary to have suitable anti-Toxoplasma sera conjugated to fluorescein isothiocyanate (F.I.T.C.). This serum does not have to be species specific therefore it is best to standardize 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.

Indirect staining:

For this method, it is necessary to have anti-Toxoplasma sera and conjugated anti-species sera. As for the direct staining method, the anti-Toxoplasma sera does not have to be species specific and may be obtained in the same way as above (i.e. sheep sera). However, the anti-species serum must be specific for the source animal species supplying the anti-Toxoplasma sera (in this case, anti-sheep sera raised in rabbits). This is conjugated to F.I.T.C. 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 is to be avoided. They may still be suitable for use after 6 months of storage but adequate controls should be employed to test them after this time).

(ii) Methods

The frozen tissues are best processed within 30 days of sampling and are sectioned at 5-8μ on a frozen microtome. The sections are air-dried for at least 20 minutes and then fixed in 10% methanol for 5 minutes at room temperature.

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

Direct Fluorescent-antibody staining:

Drops of the conjugated anti-*Toxoplasma* serum are applied to the sections and they are incubated for 30 minutes in a moist chamber at 37°C. They are then washed for 40 minutes with 5 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 counter-stained in a 1% aqueous solution of Evans Blue, mounted in phosphate-buffered glycerol (pH 9.0) and are ready for examination.

Indirect Fluorescent-antibody staining:

The sections are covered with the anti-*Toxoplasma* sera and are incubated for 30 minutes in a moist chamber at 37°C after which they are washed for 30 minutes in 4 changes of PBS. They are then overlaid with the conjugated anti-species sera and incubated for a further 30 minutes in the moist chamber at 37°C. They are then washed for 40 minutes in 5 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 are not allowed to dry out at any stage during their preparation).

Examination:

The preparations are best viewed immediately and prolonged exposure to light should be avoided as this decreases the strength of fluorescence. They are examined at 60-75 X magnification under a microscope using alternately transmitted dark-field illumination (tungsten lamp) and incident ultraviolet illumination (mercury-vapour or iodine-quartz lamp) with an adequate light filtration system.

(iii) Diagnosis

Fluorescent-antibody staining allows for the detection and identification of the *Toxoplasma* antigen within the tissues, which is characterized by yellow-green fluorescence of the zoite's walls. Frequently nonspecific 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.

II. 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.

CULTURE

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.

(a) Mouse inoculation

(i) Materials

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

White mice from a colony shown to be free of natural toxoplasmosis are used, preferably between 4 and 10 weeks of age.

(ii) Methods

Sample preparation:

Tissues and organs are finely ground with sterile sand and sterile 0.85% saline (containing 100 units of penicillin and 10 µg streptomycin per millilitre), in a glass tissue grinder and made up to a 10-20% emulsion with the same diluent. Fibrous organs and muscle are best subjected to digestion by a pepsin-hydrochloric acid 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 1 hour prior to inoculation.

Inoculation;

Each prepared sample is injected in 1 ml doses intraperitoneally or subcutaneously into 2 or 3 white mice. The former route is more desirable, but the latter is preferred for badly contaminated material. Alternatively 0.05 ml doses may be injected intracereberally.

(iii) Diagnosis

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. Trophozoites can be readily detected in peritoneal exudate and assume their characteristic appearance (crescent shaped and measuring 4-8μ in length and 2-4μ 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. Squash preparations of the brain are the most convenient means of detecting chronic infections, and characteristic tissue cysts and terminal colonies are readily detected upon examination by light microscopy at 20-40 X magnification. For the most accurate results, blood should also be collected from each mouse for serological testing for antibodies against Toxoplasma.

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.

(b) Egg inoculation

(i) Materials

Any body tissue or fluid may be tested for toxoplasmosis. Embryonated fowl eggs are used on the 10th day of incubation.

(ii) Methods

Sample preparation: Prepared as in (a) (ii).

Inoculation: 0.2-0.5 ml of each prepared sample is inoculated onto the chorioallantoic membrane (CAM) of a fertile egg using standard techniques.

(iii) Diagnosis

Pocks appear on the CAM at 7-8 days for virulent strains and at 10 days for avirulent strains. Organisms are demonstrable in these lesions when viewed unstained at 40-60 X magnification under a light microscope.

(c) Tissue culture

(i) Materials

Any body tissue or fluid may be tested for toxoplasmosis.

Monolayer cultures of HeLa cells are prepared from established cultures by trypsin-versene dispersion, and are cultured without rolling for 7 days in growth medium (medium 199 brought to pH 7.2 with $NaHCO_3$ plus 10% foetal calf serum). Other cell lines are suitable for use, the main criteria being their availability within the laboratory.

(ii) Methods

Sample preparation: The sample is emulsified in the standard diluent (medium 199 at pH 7.2) with antibiotics added (100 units of penicillin and 10µg streptomycin per millilitre).

Inoculation: The growth medium is removed after 7 days of stationary culture, and the sample emulsion and maintenance medium (medium 199 at pH 7.2 plus 1% foetal calf serum) is added. Culture is then continued at 35°C.

(iii) Diagnosis

This method is only suitable for virulent strains, which may be detected 9-11 days after inoculation. The organisms may be found in the maintenance medium or on Giesma stained cover-slip preparations.

III. DETECTION OF ANTIBODIES TO TOXOPLASMA

The diagnosis of toxoplasmosis is seldom made by the detection or recovery of the organism, for often suitable specimens for histology or culture are not available. Thus recourse is made to the detection of antibodies to Toxoplasma by various immunoserological procedures. These procedures are best employed in screening programmes 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.

IMMUNOSEROLOGY

Many immunoserological tests have been applied to toxoplasmosis, but only a few 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)*, the methylene-blue Dye Test (DT) and the Complement Fixation Test (CFT).

The IFAT and IHAT can utilize commercially available freeze-dried antigens which are adequately controlled and standardized, and present no hazards in the laboratory. The DT requires the use of a living Toxoplasma antigen and as this is time-consuming in production and may be extremely dangerous in inadequate surrounds, it is not adapted for use in the average laboratory. The CFT involves the maintenance of the organism by laboratory culture and there may be a lot of hazardous work involved in the preparation of antigens which prove to be faulty.

DT 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. CFT antibodies usually appear late in the course of active infection, generally at a time when the DT titre is high and stable. A rising CFT titre under these circumstances is valuable in demonstrating acute infections. However, as the CFT titre usually becomes negative within a few years of infection, it is unsuitable for the diagnosis of chronic infections. IHAT antibodies appear slightly later than those detected by the DT, and they persist at elevated levels for many years. IFAT antibodies appear to parallel those of the IHAT both in titre and duration.

* The technique for the IHAT for toxoplasmosis is not reported here. The test, and all relevant information, is commercially available in kit form.

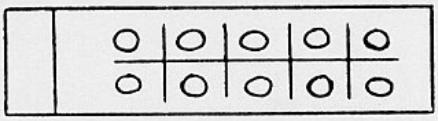
(a) The Indirect Fluorescent-Antibody Test (IFAT)

This technique is basically the labelling of an antibody-antigen aggregate 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 ultra-violet illumination. Obtaining the highest titre at which significant fluorescence last occurs enables quantitation of the test.

(i) Materials

Antigen:

Toxoplasma trophozoites are passaged at 3-4 day intervals in the peritoneal cavities of young white mice from a colony known to be free of natural toxoplasmosis. The exudate is diluted approximately 10 times in 1% formol-saline and the suspension is drawn in and out of a fine bore needle (e.g. 26G) to disperse the clumps of organisms and thoroughly mix the suspension. This is allowed to stand at room temperature for 30-60 minutes and is then lightly centrifuged for 5 minutes at 30g to remove host cells and clumped parasites. The individual organisms remaining in the supernatant are packed by centrifugation for 5 minutes at 400g, resuspended in saline and then centrifuged again for 5 minutes at 400g. The trophozoites are then diluted in saline to give approximately 20 organisms per high power field (60X). Drops of this antigen are then placed on pre-ruled, clean glass slides (as below) using a 3 mm bacteriological loop.



The slides are air-dried, fixed in 10% aqueous methanol for 1 minute, and may then be stored at -20°C for up to 2 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.

Conjugated immunoglobulins:

For the IFAT, it is necessary to have conjugated anti-species immunoglobulins which are specific for the various test animal species. Anti-sera 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 (see reference on page 3). They are best stored in concentrated aliquots at -20°C and may keep for up to 6 months.

Alternatively, conjugated immunoglobulins may be obtained commercially, but only certain anti-species are available at present.

Diluent:

Phosphate-buffered saline (PBS) (pH 7.2) is used throughout the IFAT and is prepared as follows:

NaCl	8.500 gm.
Na_2HPO_4	1.280 gm.
NaH_2PO_4	0.156 gm.
Distilled water to	1 litre.

Mounting media:

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

$NaHCO_3$	0.0729 gm.
Na_2CO_3	0.016 gm.
Distilled water to	10 ml.
Glycerol to	100 ml.

(ii) Methods

The IFAT:

The test sera are diluted in PBS in double-dilutions (usually 1:4, 1:8, 1:16, ... 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 minutes after which they are washed for 30 minutes in 3 changes of PBS. Each antigen area is then overlaid with the appropriate conjugated immunoglobulin and the slides are incubated for a further 30 minutes in the moist chamber at 37°C. The slides are washed again for 30 minutes in 3 changes of 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. It is important that the slides are not allowed to dry out any any stage during their preparation).

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.

Examination:

The preparations are positioned under a microscope at 60-75 X magnification using transmitted dark-field illumination (tungsten lamp source). The test is then read using either transmitted or incident ultra-violet illumination (mercury-vapour or iodine quartz lamp source) with a suitable light filtration system.

(iii) Diagnosis

A positive result is obtained when the Toxoplasma trophozoites 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 end-point of the IFAT is given as the highest titre at which fluorescence last occurs.

With most species, a titre of 1:16 is usually regarded as indicative of infection. This criterion holds for man 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.

(b) The Methylene-blue Dye Test (DT)

The DT depends on the principle that Toxoplasma antibody and an accessory factor (a complement-like plasma factor) modify living Toxoplasma trophozoites so that they fail to stain with methylene-blue at pH 11. Trophozoites which have not been modified by antibody stain readily, and the test is quantitated by finding the highest serum dilution which will modify 50% of the toxoplasms in a standard suspension. The DT is very sensitive and functions on most species, but is not entirely reliable on certain avian species.

Considerable experience is required with the DT and it is generally recognized that the IFAT is more practical and reproducible.

(i) Materials

Antigen:

The source of the strain of Toxoplasma used as antigen does not appear to influence DT titres. Toxoplasma trophozoites are maintained by intra-peritoneal passage in white mice at 3-4 day intervals. When antigen is required, peritoneal exudate is harvested from infected mice as closely as possible to 64 hours post-inoculation. This is diluted to produce approximately 30-80 trophozoites per 400 X microscope field, and this constitutes the antigen.

Accessory factor:

This is plasma from a human donor who is completely devoid of DT antibodies. Species other than man contain an anti-Toxoplasma factor which renders them unsuitable. Serum is totally unsuitable as accessory factor, therefore prospective donors must be bled into 3% sodium citrate in the ratio 4 parts blood to 1 part citrate. The citrated plasma remains suitable for at least 3-5 months when stored at -20°C or lower, but less than 24 hours at room temperature. Repeated thawing and freezing of the plasma is to be avoided.

Methylene-blue indicator:

The original formula of Sabin and Feldman (1948) is used, i.e.

Solution A	Sodium carbonate (anhydrous)	0.53g
	distilled water to	100 ml
Solution B	Sodium tetraborate	1.91g
	distilled water to	100 ml
Solution C	Saturated methylene-blue in 100% ethyl alcohol	

For use, the following mixture is prepared each day.

Solution A	9.73 ml
Solution B	0.27 ml
Solution C	1.0 ml

Inactivation of sera:

DT titres in man and mouse, for practical purposes, do not vary following inactivation. Other species sera usually exhibit higher titres when not inactivated than when inactivated. Inactivation conditions required for some species, to produce a stable titre which will not continue to fall on further inactivation, are as follows:-

Cats	56°C for 60 minutes
Cattle	56°C for 60 minutes
Dogs	58°C for 30 minutes
Pigs	60°C for 30 minutes
Sheep	60°C for 60 minutes

Diluents:

All serum and antigen dilutions are made in normal or complement fixation saline.

(ii) Methods

The Dye Test:

The test sera are diluted with normal or complement fixation saline into two-fold dilutions and the following preparation is made for each serum dilution.
1 part serum dilution
2 parts accessory factor
1 part prepared antigen
This is mixed and incubated in a water bath at 37°C for 60 minutes, after which time 2 parts of indicator are added. The test may then be read at once if so required.

Controls:

Two controls are required to be run concurrently with the test. Firstly, a positive control which is serum of known DT titre. A decreased titre with this control suggests either a drop in accessory factor potency or an inaccurate water bath temperature. Secondly, a negative control which consists of 2 parts accessory factor, 1 part diluent and 1 part antigen which is incubated along with the test sera. This control must give at least 90% stained (DT negative) trophozoites. If the result is below this range, the trophozoites used as antigen were probably harvested too late after the 64 hour period given earlier.

Examination:

The test is read at 40 X magnification on a light microscope with low illumination, and fitted with a blue filter to enable DT positive trophozoites to be more readily seen.

(iii) Diagnosis

The morphological changes that occur in the trophozoites used in the DT are divided into two distinct types.

DT negative trophozoites: These take up the methylene-blue indicator resulting in a trophozoite that is deeply blue staining and oval in shape. These indicate the lack of Toxoplasma antibody acting on the trophozoite.

DT positive trophozoites: These fail to take up the methylene-blue indicator resulting in an almost colourless, thin, crescent shaped trophozoite, usually with a blue staining nucleus. These indicate the action of Toxoplasma antibody on the trophozoite.

The ratio of both DT positive and negative trophozoites at a particular serum dilution is assessed. A majority of DT negative trophozoites indicates a negative result, while a majority of DT positive trophozoites signifies a positive result. A 50:50 ratio is regarded as a borderline (±) result.

The titre is given as the sum of the serum dilution plus the reagents, excluding the indicator which plays no part in the reaction (e.g. an initial serum dilution of 1:4 becomes, after the addition of accessory factor and antigen, a final dilution of 1:16).

(c) The Complement Fixation Test (CFT)

The CFT is based upon the disappearance of the haemolytic activity of complement for sensitized red blood cells when an antibody-antigen aggregate forms in its presence. When antibody is absent, the complement is left unbound and instigates damage to the red blood cells resulting in spontaneous lysis. The test is quantitated by titrating the test sera to a stage where 50% haemolysis occurs.

(i) Materials

Antigen:

Toxoplasma trophozoites are maintained by serial passage on the chorioallantoic membranes (CAM's) of embryonated fowl eggs which are inoculated on the 11th day of incubation. When antigen is required, infected CAM's are harvested at 7 days post-inoculation and are homogenized in 1 ml of isotonic saline per membrane. This is frozen and thawed 3 times and then centrifuged at 30,000G for 90 minutes. The supernatant constitutes the antigen and is standardized by carrying out a chequer-board titration with a known positive serum to give its highest working dilution. This antigen may be stored for up to 12 months at -20°C.

It is also desirable to prepare uninfected egg antigen by the same method to act as an antigen control.

Complement:

Suitable complement occurs in guinea-pig serum and the best is derived from 4-6 month old males. The serum is preserved in Richardson's solution in the ratio of 8 parts serum to 1 part solution B followed by 1 part solution A. The stock solutions A and B may be kept indefinitely and are prepared as follows:

Solution A		Solution B	
Boric acid	0.93 g	Borax	0.57 g
Borax	2.29 g	Sodium azide	0.81 g
Sorbitol	11.74 g	Saturated NaCl solution to 100 ml	
Saturated NaCl solution to 100 ml			

The resulting preserved complement may be stored for up to 2 months at 5°C. Prior to use, 1 part of preserved complement is mixed with 8 parts of distilled water to give a complement dilution of 1:10, and this is titrated with diluent to give the dilution at which 50% haemolysis occurs. The complement is then used in the CFT at the strength of 2 minimum haemolytic doses (MHD's). This solution must be prepared fresh each day and is best used within 1 hour of preparation.

Indicator system:

The indicator system consists of a suspension of 3% sheep red blood cells in haemolysin. The cells are taken from citrated sheep blood and are prepared by washing 3 times in diluent between centrifugation at 1,000 G for 10 minutes. The haemolysin is titrated to determine the concentration which, when added to an equal volume of 3% red cells, will produce an optimally sensitized suspension (usually 1.5 MHD haemolysin). The indicator system is prepared fresh each day and must be allowed to stand for at least 1 hour prior to use.

Inactivation of sera:

All test sera are inactivated at 58°C for 30 minutes immediately prior to testing.

Diluent:

Normal or complement fixation saline may be used as diluent but it must be standardized for each complete test.

(ii) Methods

The CFT:

After inactivation the test sera are diluted with saline to give double-dilutions (usually 1:4, 1:8, 1:16, and 1:32). The CFT is then performed using either macro-volumes (units of 0.25 ml) in Wasserman tubes or micro-volumes (units of 0.025 ml) on microtitre plates.

One part of each diluted test serum is added to 1 part of antigen and 2 parts of complement. This is thoroughly mixed and is fixed at 5°C overnight (for at least 12 hours). The solutions are then warmed for 10 minutes in a water bath at 37°C and 2 parts of the indicator system are then added vigorously. This is incubated for 30 minutes at 37°C and the CFT is then ready for reading.

Controls:

Positive and negative serum controls are employed to test the validity of the CFT. Also, normal antigen controls and serum controls are performed to check the antigen and to test the sera for anticomplementary behaviour.

(iii) Diagnosis

The CFT's are read using 50% haemolysis as the end-point and the result is expressed as the titre corresponding to this point. A titre of 1:4 is usually regarded as significant in most species.

The CFT is not suitable for detecting chronic infections for the antibody level declines rapidly after the first few months of infection. It also does not readily detect acute infections in poultry and swine. Marsupial sera are usually anticomplementary therefore the CFT is of limited value in these animals.