Chapter 6

TOXOPLASMOSIS

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6.1 SUMMARY

Toxoplasmosis, a zoonotic infection caused by the protozoan parasite, *Toxoplasma gondii*, has social and economic relevance to both animal and human medicine. Toxoplasmosis is a serious cause of abortion in sheep and goats in many countries of the world. Direct diagnosis of *Toxoplasma* infection is limited to isolation by inoculation of infected tissues into laboratory mice and identification of the organism in sections of placental cotyledons and/or brain of aborted foetuses. However, due to the irregular and sparse distribution of organisms in tissues the use of indirect diagnostic tools such as gross pathology, histopathology and serology are essential in the demonstration of infection.

6.2 INTRODUCTION

Toxoplasma gondii is a cyst-forming coccidian parasite of domestic ruminants world-wide. The parasite is classified in the phylum Apicomplexa, class Sporozoasida, order Eucoccidiorida and family Sarcocystidae.

The vast majority of natural *Toxoplasma* infections in domestic animals are subclinical. Clinical signs, when present, are generally vague and non-specific and may include a period of fever, anorexia, respiratory distress and sometimes diarrhoea. Central nervous system disorders are rarely recorded. *T.gondii* infection, however, is a major cause of ovine and caprine abortion and perinatal mortality.

The life cycle of *T.gondii* can be divided into two parts: an asexual cycle in intermediate hosts and a sexual cycle, confined to the enteroepitheal cells of the feline definitive host, which results in the production of oocysts. In the asexual cycle there are two developmental stages, the rapidly multiplying tachyzoite (syn. endozoite, trophozoite) and the slowly multiplying bradyzoite (syn. cystozoite). Tachyzoites actively penetrate host cells, particularly those of the reticuloendothelial system where within a parasitophorous vacuole they multiply until the cell ruptures to release organisms locally and into the bloodstream to parasitise further cells. This process continues until the host dies, or more usually it develops immunity, extracellular parasites are then eliminated, intracellular multiplication slows, tissue cysts containing bradyzoites develop and a chronic or persistent infection is established. These microscopic cysts are found most frequently in brain and skeletal muscle and represent the quiescent stage of the parasite within the host. In some species, such as sheep, goats, pigs and man, cysts may remain for the rest of the life of the individual, while in others such as cattle, water buffalo and deer the host may eventually become clear of infection.

The sexual cycle is initiated when a non-immune cat ingests infective *T. gondii* either in the form of tissue cysts, tachyzoites or oocysts. The most impor-

tant sources of feline infection are birds and rodents persistently infected with tissue cysts. Gastro-intestinal proteolytic enzymes release bradyzoites from tissue cysts to penetrate the epithelial cells of the cat's small intestine, where subsequent sexual reproduction results in the formation of oocysts, which are released into the gut lumen to be excreted in the faeces. The prepatent period after ingestion of tissue cysts is 3-6 days and vast numbers of oocysts may be shed continuously in the faeces from this time until up to 14 days after infection. Following sporulation in the open over the next one to five days, depending upon aeration and temperature, the oocysts become infective. They are very resistant and may remain infective in the environment for a year or more. Sporulated oocysts are 11 x 13 mm in diameter and contain four sporozoites in each of two sporocysts.

The major source of *Toxoplasma* infection for susceptible herbivores is feed and water contaminated with sporulated oocysts. Fifty grams of infected cat faeces may contain as many as ten million oocysts. If, in a hypothetical situation this was evenly distributed throughout ten tonnes of concentrated animal feed, then each kilogram could contain between five and twenty-five sheep-infective doses. Similarly, pasture spread with manure and bedding from farm buildings where cats live may be a source of infection for sheep.

To limit environmental contamination by oocysts the number of cats capable of becoming infected and shedding oocysts should be reduced. Since these tend to be young animals, breeding should be controlled to favour a small healthy population of mature animals. Feed should also be kept covered to prevent its contamination by cat faeces.

Abortions and neonatal mortality occur when sheep and goats suffer a primary infection during pregnancy. Within four days of ingestion of sporulated oocysts by susceptible pregnant sheep, organisms can be found in the mesenteric lymph nodes, where they multiply causing marked lymph node enlargement, sometimes with focal necrosis. Around the fifth day tachyzoites are released to cause a parasitaemia which may last until the twelfth day. Coinciding with the parasitaemia the ewe displays a febrile response which can exceed 41°C around day six or seven. The cessation of the parasitaemia coincides with the onset of an effective maternal immune response. With the exception of the gravid uterus, the infection then persists as bradyzoites within tissue cysts.

In pregnant ruminants the gravid uterus is an "immunologically privileged" site. On the uterine side maternal immunological responses are locally suppressed, while the ability of the foetus, with its placenta, to recognise and respond to a pathogen such as *T.gondii* commences during the first half of gestation and develops for the remainder of pregnancy, so that foetal lambs become immunocompetent by the time they are born. During maternal parasitaemia, tachyzoites are able to parasitise the caruncular septa, invade the adjacent trophoblast cells of the foetal villi, and from there the rest of the foetus, between five to ten days after the onset of parasitaemia.

In a typical outbreak, disease first becomes apparent when ewes lamb a few days early with a significant proportion of lambs, although outwardly quite normal, being stillborn and some accompanied by a "mummified" foetus. In addition a proportion of lambs born alive are weak and

die within the first few days of life despite careful nursing. The ewe remains clinically normal. However, the real effect of infection in susceptible pregnant ewes depends upon the stage of gestation at which infection occurs. Infection in early pregnancy, when there is a minimal foetal immune response, rapidly results in foetal death followed by foetal resorption with the ewes ending up barren. In naturally-occurring outbreaks of toxoplasmic abortion aborted foetuses are seldom detected before the fourth month of gestation. Infection between about 70 and 120 days presents the clinical picture described while ewes which become infected in late pregnancy would be expected to produce infected but clinically normal lambs. The parasite does not cause abortion in sheep in subsequent pregnancies following toxoplasma abortion or if ewes are first infected whilst not pregnant. The key to preventing uterine infection is to prevent parasitaemia occurring during pregnancy.

No killed *Toxoplasma* vaccines are currently available for use in animals or man. However, a vaccine containing a live attenuated strain of *T.gondii* (S48) is available in New Zealand (Toxovax; MAF) and some European countries (Toxovax; Intervet/Mycofarm) for use in sheep. Live tachyzoites are injected into sheep where they induce a short-lived infection. Thus, while sheep are protected they do not appear to be left persistently infected, so avoiding the potential public health hazard of meat persistently infected with a vaccinal strain of *T.gondii* being available for human consumption. Correctly used the vaccine is very effective, its disadvantages are that is has a very short shelf-life and if not handled with care it could be a hazard to those using it or handling meat from animals killed soon after vaccination.

The ionophore, monensin, which has significant anti-toxoplasma activity in sheep, may be used to control infection in countries where legislation permits its use. The anticoccidial drug, decoquinate, has also recently been shown to significantly reduce the effect of *Toxoplasma* oocysts ingested by pregnant sheep. Both monensin and decoquinate work best if they are already being fed to susceptible ewes at the time they first encounter infection. They have not been shown to be so effective as chemotherapeutic agents. Administration of certain drug combinations such as pyrimethamine and sulphamezathine after infection has become established in the placenta and foetus may be effective. However, since the first abortions in a flock occur several weeks after infection, the practical benefit of chemotherapy is limited in many instances.

6.3 SAMPLES

Diagnosis of *Toxoplasma* infection is generally carried out using one or more of the following samples:

- Placental material
- Brains from aborted foetuses
- Foetal fluids
- Blood :Blood from aborted ewes · Pre-colostral blood from live lambs

6.3.1 Isolation

Toxoplasma gondii can be isolated from placental cotyledons, the brain, pleural and peritoneal fluids and heart blood from aborted foetuses that are not too autolysed. Samples should be collected as soon as possible after abortion and expulsion of the placenta as described in Chapter 2. If not analysed immediately, samples should be stored frozen at -20°C.

6.3.2 Histology

Five or six cotyledons and the entire brain from aborted foetuses that are not too autolysed should be aseptically sampled and placed in a glass sample jar containing a suitable fixative as described in Chapter 2. In the absence of secondary fixation tissues are best left for at least two weeks before further processing.

6.3.3 Antibody detection

Blood samples from aborted ewes are best collected at the time of abortion when antibody titres to *Toxoplasma gondii* would be expected to be raised. Precolostral blood samples from lambs born weak or lambs born alongside aborted foetuses can sometimes aid a diagnosis, however, there are practical difficulties in obtaining such samples.

Blood samples for serological examination should preferably be collected in sterile, evacuated glass tubes (e.g. Vacutainers) and removed to the laboratory as quickly as possible. Samples should be centrifuged for 10 minutes at 3,000 xg, sera removed from the clot and either analysed immediately or stored frozen at -20°C.

6.4 RISKS TO HUMAN HEALTH

Toxoplasma is classified as a category 1 pathogen. Samples must be collected and handled with the same precautions as all biological materials that are potentially dangerous to human health. Laboratory work should be undertaken in type L3 containment areas or, if unavailable, in a conventional laboratory equipped with a laminar flow hood.

Toxoplasma can present a serious threat to the unborn child if the mother becomes infected for the first time during pregnancy. Maternal infection acquired early and transmitted to the foetus usually results in spontaneous abortion, stillbirth or severe disease of the unborn child. The greatest risk of foetal damage occurs following maternal infection between 10 and 24 weeks of pregnancy. Maternal infection in later pregnancy most commonly results in subclinically- infected infants, although they may subsequently display severe symptoms such as mental retardation and visual handicap, the latter as a result of a progressive chorioretinitis.

Other people at risk of developing clinical illness include those who are immunosuppressed, such as tissue transplant patients and victims of AIDS, patients receiving chemotherapy for cancer and individuals with certain tumours of the lymphoid system. For such vulnerable groups, infection with *Toxoplasma* can prove life-threatening.

Emphasis in the laboratory should be placed on educating staff of the potential risks of accidental infection with this zoonotic agent. Pregnant women and other personnel knowingly in any other "at risk" category should not be allowed to handle any materials possibly infected with *Toxoplasma*. Ideally non-pregnant female staff should be screened twice yearly for antibody status to *Toxoplasma*. Pregnant women should not assist with lambing ewes or kidding goats or the care of newborn lambs or kids not only because of the risk of toxoplasmosis but also because sheep and goats may carry other zoonotic infections such as *Chlamydia psittaci* and *Coxiella burnetti*.

In the event of accidental inoculation or ingestion of potentially infective material personnel should be referred to medical experts who may opt to treat with chemotherapeutic drugs such as a course of pyrimethamine and sulphonamide.

6.5 DIRECT DIAGNOSIS

A definitive diagnosis of toxoplasmosis as a cause of abortion and neonatal mortality in sheep and goats is generally based on a combination of the identification of distinct macroscopic and histological changes and the outcome of serological tests on the ewe or foetus and/or results of isolation tests. The actual techniques used in any specific case depends upon the availability of the different types of samples.

6.5.1 Gross pathology of placenta and aborted foetuses

The most characteristic macroscopic changes following Toxoplasma abortion in sheep and goats are found in the placenta. The cotyledons are usually bright to dark red and speckled with white foci of necrosis 2 to 3 mm in diameter. These foci may be sparse or so numerous that they can become confluent and on cut section they can be seen to occur on any plane. Cotyledons with macroscopic changes can be found alongside apparently normal ones in the same placenta. The intercotyledonary allanto-chorion appears normal (Figure 6.1, page 95).

Visible changes in lambs can vary, the most obvious being the mummified foetus, a small chocolate brown miniature of a lamb, often with its own small grey-brown placenta. Foetuses dying later in gestation are born in various stages of decomposition often with clear to bloody subcutaneous oedema and a variable amount of clear to bloodstained fluid, sometimes flecked with strands of fibrin, in body cavities. However, while these latter changes indicate an intrauterine infection they are not specific to *Toxoplasma* infection.

6.5.2 Histopathological examination of tissues

6.5.2.1 Simple staining

Principle

This technique relies on showing the presence of histopathological changes in tissue sections following haematoxylin and eosin staining. In rare cases organisms of *T.gondii* can also be visualised.

N.B. The chemical concentrations and timings of the various processes involved in fixing and staining samples for histopathological examination will vary according to the availability of equipment and chemicals in the laboratory undertaking the work. Many laboratories will have their own standard methods and the method described is given as one example.

Materials and reagents

- Microtome
- Ethanol
- Toluene
- Xylene
- Paraffin wax.

Preparation of histological tissue sections

1. Prior to processing, samples of placenta must have been fixed for a minimum period of one week and brains for a minimum of two weeks. More rapid fixation can be achieved using Bouin's fixative either on its own or as a secondary fixative.

2. Remove tissue from fixative, blot dry and trim into suitably sized blocks no more than 4mm thick.

3. Dehydrate tissue blocks by gently agitating them in an alcohol series (80% ethanol for 45 minutes, 94% ethanol for 60 minutes, 99% ethanol for 3 x 60 minutes).

4. Clear blocks by immersion in a mixture of equal parts of 99% ethanol/toluene for 2 x 90 minutes then toluene alone for 2 x 60 minutes.

5. Embed blocks in paraffin wax by immersing in molten wax for 2 x 120 minutes.

6. Trim the resultant blocks and cut sections of uniform thickness between 4 and 6mm with a microtome.

7. Mount sections on glass slides pretreated with 2% egg albumin or equivalent to bind the section to the glass.

Staining

1. Immerse slides in xylene for 2 x 10 minutes to remove wax.

2. Hydrate tissue through a graded alcohol series (99% ethanol for 2 x 2 minutes, 90% ethanol for 2 minutes, 70% ethanol for 2 minutes).

3. Rinse slides thoroughly under running tap water.

4. Immerse slides in haematoxylin stain for 5-15 minutes to stain the cell nuclei.

5. Rinse slides thoroughly under running tap water.

6. Immerse slides in Scotts Tap Water Substitute (STWS) for 3 minutes.

7. Examine under a microscope: if the background is blue differentiate by immersing in 1% HCl in 70% alcohol to clear to grey; when the background is grey continue to step 8.

8. Rinse slides thoroughly under running tap water and re-immerse in STWS for 3 minutes.

9. Rinse slides thoroughly under running tap water.

10. Immerse slides in eosin solution for 3 minutes.

11. Rinse slides thoroughly under running tap water.

12. Dehydrate and clear slides through a graded alcohol series and xylene (70% ethanol for 2 minutes, 90% ethanol for 2 minutes, 99% ethanol for 3 x 2 minutes, xylene for 2 x 2 minutes).

13. Mount coverslip over stained tissue section and examine under a microscope.

Reading and interpretation of results

Cell nuclei will appear blue; red blood cells, red; muscle, connective tissue and cell cytoplasm, varying shades of pink. *Toxoplasma* will appear light blue.

Well-preserved samples of placental cotyledons can reveal moderate oedema of the mesenchyme of the foetal villi with a diffuse hypercellularity due to the presence of large mononuclear cells. In addition there can be foci of swollen trophoblastic epithelium in foetal villi which can progress to necrosis and desquamation. The larger affected areas give rise to foci of coagulative necrosis which may become mineralised with time. Sometimes small numbers of intracellular and extracellular toxoplasms are visible, usually on the periphery of a necrotic area or in a villus which is in the early stages of infection. The organisms appear ovoid, 1-4µm long, with nuclei which are moderately basophilic and located centrally or towards the blunt end [1].

In the foetal brain both primary and secondary lesions develop. Glial foci, typically surrounding a necrotic and sometimes mineralised centre, often associated with a mild lymphoid meningitis, represent a foetal immune response following direct damage by local parasite multiplication. Toxoplasms are only rarely found, usually at the periphery of the lesions. Focal leukomalacia is also common and is thought to be due to foetal anoxia in late gestation caused by advanced necrosis in the placentome preventing sufficient oxygen transfer from

mother to foetus. These foci occur most commonly in cerebral white matter cores and the corpus striatum but sometimes also in the cerebellar white matter [1].

Preparation of reagents

- Bouin's Fixative

Saturated aqueous picric acid	75ml
40% formaldehyde	25ml
Glacial acetic acid	5ml

- Haematoxylin Solution

Haematoxylin	1g
Sodium iodinate	0.2g
Potassium alum	50g
Chloral hydrate	50g
Citric acid	1g
Distilled water	1 litre

Dissolve the haematoxylin in the water with the aid of gentle heat then add the sodium iodinate and alum and shake at intervals to effect the solution of alum. Add citric acid and chloral hydrate and mix until dissolved. The solution remains for several months.

- Scott's Tap Water Substitute

Sodium bicarbonate	3.5g
Magnesium sulphate	20g
Tap water	1 litre
Thymol	1 crystal

Dissolve salts in the water and add thymol.

- Eosin Solution

Eosin	1g
Distilled water	100ml
Thymol	1 crystal

Dissolve eosin in water and add thymol.

6.5.3 Detection of *Toxoplasma gondii*

The most direct and established method of demonstrating *Toxoplasma* infection in cases of abortion is to transmit the infection from aborted material to laboratory mice [2]. The method, however, is slow and does not distinguish between acute and latent infections. *Toxoplasma* can be grown in virtually any mammalian cell line. However, although more rapid than mouse inoculation, cell culture is rarely used for routine diagnosis. An alternative, much more rapid but less sensitive, method of isolation is the direct demonstration of *T.gondii* tissue cysts, by centrifugation of lamb brain homogenate on a discontinuous density gradient of 30% and 90% colloidal silica solution [3].

Immunohistochemical techniques allowing visualisation of intact *T.gondii* and its antigenic residues in tissue sections of aborted materials are recognised as convenient and sensitive methods of demonstrating *Toxoplasma* infection. Such methods have the advantage of detecting *Toxoplasma* antigen even in severely decomposed tissues.

Techniques such as the polymerase chain reaction (PCR) for identifying and amplifying specific lengths of DNA have been developed for *T.gondii*. PCR and related techniques involving nucleic acid probes still need to be evaluated as diagnostic tools in veterinary protozoology but may become important techniques in the future.

6.5.3.1 Mouse inoculation

Principle

Isolation of *T.gondii* from dead lambs and foetal membranes are best made by inoculation of laboratory mice. The choice of material for inoculation is dictated by the quality of the material available, the best being the cotyledons and foetal brain.

Materials and reagents

- Equipment suitable for aseptic maceration of tissues e.g. a "stomacher" (Seward Laboratory, London, UK)
- Laboratory mice e.g. Swiss white
- Sterile phosphate buffered saline (PBS)
- Antibiotics: Penicillin
  Streptomycin
- Giemsa stain.

Procedure

1. Aseptically remove 2-5g of placental material or brain tissue from the dead lamb.
2. Homogenise the tissue in an equal volume of sterile PBS with added antibiotics (100 IU/ml penicillin and 745 IU/ml streptomycin) in a "stomacher" or other suitable macerating equipment. Brain tissue may be effectively macerated by passing it through a 16 gauge needle 10 times by means of a syringe.
3. Inoculate each of 3 *Toxoplasma*-free mice intraperitoneally with 0.5ml of the homogenate.
4. Six to 8 weeks after inoculation, kill the mice and remove brains. Blood should also be recovered from the mice at this stage and serum separated and stored at -20°C.
5. Homogenise each mouse brain with an equal volume of sterile PBS in a "stomacher" or by passing through a 16 gauge needle 10 times by means of a syringe.
6. Place drops of the suspensions on a suitable number of slides.
7. Dry and stain with Giemsa, dehydrate and mount under a coverslip.
8. Examine slides under a microscope.

Reading and interpretation of results

Tissue cysts appear as circular structures measuring 5 to 50µm filled with blue-staining crescent shaped bradyzoites.

Failure to demonstrate cysts does not rule out a positive diagnosis. Serum from the mice must be analysed for the presence of antibodies to *Toxoplasma* and if this analysis is also negative, *Toxoplasma* infection can be ruled out.

Preparation of reagents

- Giemsa Stain

Giemsa powder	4g
Glycerol	250ml
Methanol	250ml

Stock Solution: Dissolve giemsa powder in glycerol at 60°C with regular agitation. Add methanol, mix thoroughly and leave to stand for 7 days. Filter before use.

Working Solution: Dilute 4ml of stock solution in 96ml buffered distilled water (pH 6.8).

6.5.3.2 Immunohistochemical methods

In order to overcome problems of visualisation of *Toxoplasma* organisms in histological sections, immunohistochemical labelling methods detecting *T.gondii* antigens have become increasingly important. Two immunoperoxidase techniques are regularly used. The ABC indirect immunoperoxidase method is available commercially in kit form (VECTASTAIN" Elite ABC Kit, Vector Laboratories, USA) combined with an appropriate primary antibody. Alternatively the peroxidase anti-peroxidase (PAP) technique [4] can be carried out as follows:

Principle

The PAP method relies on the use of a primary antibody specific for the antigen in question, a "PAP complex" comprised of the enzyme peroxidase and an antibody against peroxidase, and a "link" (secondary) antibody capable of binding to both the primary antibody and to the PAP complex. The ABC and PAP method both allow visualisation of

T.gondii organisms as well as parasitic intra- and extracellular antigenic residues even in quite severely decomposed and necrotic tissues.

Materials and reagents

- Microscope.
- Staining boxes and slides.
- Xylene.
- Ethanol.
- Tris-buffered saline solution (TBSS).
- Hydrogen peroxide.
- Non-immune porcine serum (Dakopatts, Copenhagen, Denmark).
- Rabbit anti-Toxoplasma serum.
- Porcine anti-rabbit immunoglobulin (PAP) (Dakopatts, Copenhagen, Denmark).
- Substrate : Diaminobenzidine (dissolve 24mg diaminobenzidine-tetrahydrochloride in 40ml TBSS buffer and add 0.4ml 3% hydrogen peroxide immediately before use).
- Haematoxylin stain.

Procedure

All incubations should be carried out at room temperature in a humidity chamber.

1. Fix and cut tissue sections as described in Section 6.5.2.1.
2. Dewax sections by immersing slides in xylene for 2 x 2 minutes.
3. Rehydrate tissues through an alcohol series (99% ethanol for 2 minutes, 90% ethanol for 2 minutes, 70% ethanol for 3 x 2 minutes).
4. Block endogenous peroxidase activity by immersing slides in 3% hydrogen peroxide in Tris-buffered saline solution (TBSS) for 15 minutes.
5. Incubate the tissue sections for 30 minutes with 1:10 dilution of normal non-immune porcine serum.
6. Remove the excess liquid and incubate for 45 minutes with the pre-determined optimal dilution of primary rabbit anti-Toxoplasma serum.
7. Rinse slides 3 times with TBSS.
8. Remove the excess liquid and incubate for 30 minutes with a 1:20 dilution of PAP.
9. Rinse slides 3 times with TBSS.
10. Incubate slides for 30 minutes with a 1:100 dilution of PAP.
11. Rinse slides 3 times with TBSS.
12. Place slides in substrate bath containing diaminobenzidine solution and monitor the positive control wells for development of specific brown staining (up to 8 minutes).
13. Rinse slides 3 times with TBSS.
14. Counterstain slides with haematoxylin.
15. Examine tissue sections microscopically.

Reading and interpretation of results

Toxoplasma tachyzoites appear as brown, round or crescent-shaped organisms, approximately 2 x 5µm. Tachyzoites are usually situated intracellularly, singly or in clusters of 4-10 organisms. The parasite cell wall tends to be distinctly stained. Bradyzoites are similar in appearance to tachyzoites but will be seen within tissue cysts in muscle or brain (Figure. 6.2, page 95).

6.5.3.3 DNA analysis

Techniques such as the polymerase chain reaction (PCR) for identifying and amplifying specific lengths of DNA have recently been developed for T.gondii. Methods are based on the amplification of either the P30 or the B1 gene of *T.gondii* and have been used for the detection of *Toxoplasma* in various clinical specimens collected from infected humans. Limited studies have also been carried out using ovine samples and the methods have been successfully applied

to aborted placental material, brain and peritoneal fluid from aborted foetuses and lymph, blood and lymph nodes from artificially infected ewes [5]. Detection of *T.gondii* by amplification of the B1 gene has been shown to be more sensitive than by the P30 gene. This may be due to the repetitive nature of the B1 gene of which 25-50 copies are present in the genome of *T.gondii* whereas there is only a single copy of the longer P30 gene. However, further comparative investigations into the efficacy of P30 and B1 PCR in a full range of clinical materials are required before any conclusion can be made about the use of either target gene for positive detection of *T.gondii* and the use of such a method in routine diagnosis.

6.5.4 Serological methods

Serology is an important tool in the diagnosis of ovine and caprine *Toxoplasma* abortion. The presence of specific antibodies in serum or tissue fluid from stillborn lambs or kids or in precolostral serum from live lambs or kids indicates uterine infection. However, high *Toxoplasma* antibody titres in sera taken from ewes within a few weeks of abortion or production of stillborn lambs or kids can only suggest toxoplasmosis as titres remain relatively high for long periods after initial infection. Serology is very useful as a way of assessing the degree of exposure to infection in a given group of animals. Many serological methods for the diagnosis of toxoplasmosis have been established over the years. Most have been developed for detection of *Toxoplasma* infection in humans and are now commercially available in kit form. Many of the techniques are readily modified for use in the detection of *Toxoplasma* infection in other species including sheep and goats but are not produced as commercially available kits. The methods include :

a) Dye test of Sabin and Feldman (DT)
b) Complement Fixation test (CFT)
c) Indirect Fluorescent Antibody test (IFAT)
d) Direct Agglutination test (DAT)
e) Latex Agglutination test (LAT)
f) Indirect Haemagglutination test (IHA)
g) ELISA
h) Radioimmunoassay (RIA)
i) Carbon Immunoassay (CIA)
j) Diffusion-in-gel ELISA (DIG-ELISA)

6.5.4.1 Comparison of serological methods

The first methods to be developed were the DT of Sabin and Feldman and the CFT. The DT is sensitive and specific and is traditionally regarded as being the definitive test for *T.gondii* antibody detection. However, it has the drawbacks of being expensive and time consuming, requiring live *Toxoplasma* tachyzoites as antigen, and has now been replaced in many laboratories by less demanding methods.

The IFAT gives titres comparable with the DT and is extensively used for the analysis of ovine and caprine sera. It is a safer technique than the DT for the operator as it uses killed tachyzoites but a fluorescent microscope and skilled staff are required for the reading and interpretation of results, which renders the IFAT suitable primarily for well equipped central laboratories. Antigen for use in IFAT analysis for sheep and goats is commercially available from BioMerieux, France.

The IHA and LAT tests are easy to perform and both are commercially available in kit form (Tox HA Test; Wellcome Diagnostics, UK: Toxoreagent Kit; Eiken Chemical Co., Japan distributed by Diamed Diagnostics, Bootle, UK). IHA and LAT tests do not require species-specific antisera or conjugates and since they are available in kit form they have become popular for serodiagnosis of ovine toxoplasmosis. However, antibodies detected by IHA and LAT may appear later in infection than those detected by tests such as DT and IFAT and their sensitivity may therefore be low especially in acute infections.

ELISA for *T.gondii* antibodies has been adapted for use in most domestic animals including sheep and goats and modified methods have been developed for the detection of *Toxoplasma* antigen in body fluids. The ELISA is readily automated and therefore suitable for handling large numbers of test sera and can also distinguish IgG and IgM antibodies.

The RIA, CIA and DIG-ELISA techniques are less commonly used for *Toxoplasma* antibody detection in animals.

6.5.4.2 Indirect fluorescents antibody test (IFAT)

Principle

Antibodies to *T.gondii* present in test samples are bound to antigen fixed on microscope slides. Immunoglobulin bound in this way is detected by subsequent binding of fluorescently labelled antiglobulin [6].

Glass microscope slides with 10 printed circular areas covered with RH strain of *T.gondii* may be obtained from Gull Laboratories, Salt Lake City, USA. Alternatively, lyophilised formalin treated antigen slides can be purchased from BioMerieux, France.

Materials and reagents

- Humidity chambers.
- Fluorescent microscope.
- Antigen slides.
- Fluorescein isothiocyanate (FITC) conjugated rabbit anti-sheep immunoglobulin (Dakopatts, Denmark).
- Standard positive and negative sera.

Procedure

1. Dilute test and standard positive and negative sera in PBS (pH 7.6) 1:10 and subsequently in twofold steps.
2. Add one drop of each serum dilution to prepared antigen wells on microscope slides.
3. Incubate slides at room temperature in a moist chamber for 30 minutes.
4. Wash slides twice using PBS (pH 7.6) and air dry.
5. Cover antigen with a suitable dilution of FITC-labelled rabbit anti-sheep IgG preparation and incubate at room temperature in a moist chamber for 30 minutes.
6. Wash slides twice using PBS (pH 7.6) and air dry.
7. Cover antigen using a coverslip and buffered glycerol (pH 8 - 9).
8. Read slides by fluorescent microscope at a final magnification of between 200 and 400.

Reading and interpretation of results

A sample dilution is considered to be positive when at least 50% of the tachyzoites show a bright unbroken peripheral fluorescence. However a titre is not normally considered to be positive in adult sheep and goats unless it is equal to or greater than 1/160 while in foetal samples a titre of 1/40 or more is likely to represent a specific response to the parasite.

6.5.4.3 ELISA

Principle

Test sera are reacted with *T.gondii* antigen-sensitised wells of a microtitre plate. Bound antibody is then detected by addition of an enzyme-linked antiglobulin followed by an assay of the enzyme reaction with its substrate [7].

A commercially available kit (Toxoelisa; Microbiological Associate Bioproducts, USA) for use in the diagnosis of human *Toxoplasma* infections can be used for ovine samples by replacing the anti-human IgG conjugate with an alkaline-phosphatase labelled rabbit, anti-sheep IgG (Kirkgaad and Perry Laboratories Inc., USA) or antigen coated plates can be obtained from BioMerieux, France and the test carried out as follows :

Materials and reagents

- ELISA plate reader.
- *Toxoplasma* antigen coated microtitre plates (BioMérieux, France).
- Phosphate buffered saline (PBS).
- Tween 20.
- Horseradish peroxidase (HRP) conjugated rabbit-anti-sheep immunoglobulin (Dakopatts, Denmark).
- Substrate: 1 mg p-nitrophenylphosphate per ml diethanolamine buffer, pH 9.8, containing 0.5 mmol 2M $MgCl_2$.
- Standard positive and negative sera.

Procedure

1. Dilute test and standard sera samples 1 in 400 with PBS with 0.05% Tween 20.
2. Add 100µl of diluted serum to each well and incubate at room temperature for 2 hours.
3. Wash the plates three times each for 3 minutes in PBS with 0.05% Tween 20 and shake dry.
4. Add 100µl of the HRP conjugated rabbit anti-sheep immunoglobulin diluted to a suitable concentration in PBS-Tween 20 to each well and incubate at room temperature for 3 hours.
5. Wash the plates three times each of 3 minutes in PBS with 0.05% Tween 20 and shake dry.
6. Add 100µl of substrate to each well and leave for 30 minutes.
7. Stop the reaction by addition of 50µl 2M sodium hydroxide to each well.
8. Read the optical density (O.D.) of each well at 400nm.

Reading and interpretation of results

Antibody can be expressed either as a titre or as a percentage of a standard positive serum. A sample of Toxoplasma-positive serum, which consistently produces an O.D. in the region of 1.0 is used at different dilutions in an ELISA to construct a standard curve. The O.D. at a dilution of 1 in 500 is read and given the arbitrary value of 100% and all subsequent O.D. values within the run are expressed as a percentage of this with the equation :

$$\frac{\text{(Experimental O.D.) - (Negative antigen O.D.)}}{\text{(Standard O.D.)-(Negative antigen O.D.)}} \times \frac{100}{1}$$

6.6 FUTURE WORK

The diagnosis of toxoplasmosis is well established with numerous diverse immunological techniques suitable for many situations. To standardise and further increase their specificity, defined antigens and antisera are required. In the future, early diagnosis of toxoplasma infection in sheep and goats with developing placental pathology could allow the employment of chemoprophylactic and chemotherapeutic regimes. The detec-

tion of parasite DNA by nucleic acid probe techniques such as the PCR also needs to be evaluated. Their specificity and high sensitivity will ensure them an important role in veterinary diagnosis.

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