Laboratory techniques in the investigation of toxoplasmosis

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Introduction
Infection by the protozoan *Toxoplasma gondii* is one of the most common parasitic infections of warm-blooded animals including man. The definitive host is the cat in which the sexual life cycle takes place. Human infection may arise in utero or by ingestion of cysts in undercooked meat or by ingestion of oocysts in soil or via contaminated foods. Seroconversion increases with age and varies according to geographical area and eating habits. Reported prevalence rates, as determined from the presence of serum antibodies, are 20–40% in Great Britain, 50–60% in the USA and 80–90% in France.12

Acute toxoplasmosis is well recognised as an important opportunistic infection of the immunocompromised.3 Cancer patients, organ-transplant recipients and patients with the acquired immune deficiency syndrome (AIDS) are all at risk of severe, sometimes fatal, toxoplasmosis. The infection in these patients has a predilection for the central nervous system (CNS).3 Since the recognition of AIDS, toxoplasmosis has become one of the most common causes of encephalitis in the USA.45 It is the most common cause of intracerebral mass lesions in patients with AIDS and is possibly the most common opportunistic infection of the CNS.

Infection of the central nervous system (CNS) in AIDS patients varies enormously and probably reflects prevalence of parasitic infection in different populations. Involvement of other organs is rare but pulmonary toxoplasmosis is said to occur in 1% of AIDS patients7 and a diffuse retinochoroiditis has been recorded.8

The early diagnosis of CNS toxoplasmosis in AIDS patients requires a high index of suspicion. The patient may present with focal or generalised neurological abnormalities. Computed tomography (CT) is extremely useful for investigating suspected CNS toxoplasmosis. Lesions, single or multiple, and isodense or hypodense are usually seen in the cerebral hemispheres often with surrounding oedema and mass effect (fig). Contrast studies reveal either ring or nodular enhancement in most cases. Magnetic resonance imaging, if available, may detect lesions not demonstrated by CT. Most often a diagnosis is made in AIDS patients on the basis of compatible clinical, radiological and serological findings which is only confirmed after a clinical and radiological response to anti-parasite therapy. This article reviews the laboratory techniques available for investigating toxoplasmosis with particular reference to patients with AIDS. These techniques include serology, histology, culture and specific nucleic acid detection by DNA probe and the polymerase chain reaction (PCR). Clinical and radiological findings will not be discussed further.

Serological techniques
Detection of toxoplasma specific antibody in serum is the investigation of choice in the immunocompetent patient. Rising IgG titres and demonstrable IgM may be seen in cases of acute toxoplasmosis but often the diagnosis is not considered until late in the illness and it is the persistence of specific IgG which confirms toxoplasma exposure at some time. In the majority of cases toxoplasmosis in AIDS is associated with a secondary reactivation of a
chronic, previously latent, infection. Primary infections with seroconversion, rising titres and specific IgM production are thought to be rare, although in a recent study in this country 10 of 20 documented cases of CNS toxoplasmosis had demonstrable IgM by one or more assay. However, the absence of specific IgG antibody in the peripheral blood of AIDS patients excludes the diagnosis of CNS toxoplasmosis. Antibody tests are of two major types depending on the antigen source used. Assays incorporating whole organisms include the Sabin and Feldman dye test, direct agglutination test and immunosorbent agglutination assay. These predominantly detect antibodies to membrane antigens and are most reactive in the early stages of infection. Cytoplasmic antigens are exposed by disrupting the trophozoites and assays using this antigen source (complement fixation test, enzyme linked immunosorbent assay, latex agglutination and haemagglutination tests) are slower to become positive in the course of the infection but remain reactive for a greater length of time. Antibody levels do not correlate with severity of illness. Whilst many laboratories perform a latex agglutination test on serum samples, confirmation of IgG and IgM positivity and the investigation of the immunocompromised and acute infection during pregnancy is best performed by reference centres. Assays commonly used by such laboratories will be discussed further.

The Sabin and Feldman dye test. This is the accepted reference assay for detecting toxoplasma specific antibody. The dye test uses live, virulent trophozoites and is therefore not performed outside of reference laboratories. Bound antibody fixes complement which then damages the parasite cell wall preventing the organism from retaining the vital stain alkaline methylene blue. Titres are expressed in international units as compared with a reference serum. Unlike other assays, the dye test detects both complement fixing IgG and IgM. Although not easy to perform the dye test is both highly sensitive and specific. Patients become dye test positive within 1–2 weeks of exposure, reaching a peak by 8 weeks. Low titres commonly persist for life.

Direct agglutination assay
First described by Fulton and Turk, and involving the reaction of specific antibody with formalin-fixed trophozoites to form a visible agglutination pattern, this assay is still widely used. Sensitivity and specificity have been improved by pre-treatment of trophozoites with trypsin to expose additional antigens and removal of non-specific IgM respectively. Using the dye test findings as a reference one recent study found the sensitivity and specificity of the direct agglutination assay to be 96% and 98% respectively.

Having established the presence of toxoplasma specific antibody in the peripheral blood, thereby identifying those AIDS patients at risk of reactivation, diagnosing those with reactivated cerebral infection by serological means alone is difficult. It has been suggested that calculating direct agglutination to dye test titre ratios may be of value. In one study 12 of 16 patients with AIDS and CNS toxoplasmosis had ratios greater than 20, whilst in normal individuals with comparable dye test titres the ratio was less than 10. A subsequent report suggests a ratio $\geq 5$ is indicative of CNS toxoplasmosis although lower values do not rule out the diagnosis.

The agglutination test may also be performed using acetone rather than formalin fixed trophozoites. Titres in the two agglutination reactions have been compared and when used together had a sensitivity and specificity of 70% and 93% respectively for the diagnosis of CNS toxoplasmosis in a group of 43 biopsy-proven cases.

Latex agglutination tests
The latex agglutination test is widely used in screening for toxoplasmosis. Antigens derived from disrupted trophozoites are fixed to a latex bead and when serum containing specific antibody is added visible agglutination occurs. This test will not detect antibody for several weeks into the course of an infection reflecting the predominantly cytoplasmic antigen source.

False positive reactions do occur with the latex agglutination test when compared with dye test findings. In one analysis of 4450 sera there were 59 (1.3%) such discrepant latex agglutination test results. These false positives were associated with an unspecified IgM antibody but not with CMV infection or hepatitis B virus "e" antigen as suggested by other workers. False negative reactions are uncommon. One study found the sensitivity and specificity of the latex agglutination test to be 99% and 81% respectively when using the dye test as a standard reference. Whilst there is no evidence that false positive and false negative results are more common in the AIDS patient, misleading results have been reported in heart transplant recipients. It is advisable to investigate the immunocompromised by additional serological tests.

Enzyme linked immunosorbent assay (ELISA)
ELISA technology is becoming increasingly popular as a means of microbiological diagnosis and a number of such assays are available on a commercial basis for detection of toxoplasma specific antibodies. The sensitivity of conventional ELISA methods can be improved by the use of double sandwich assays (DS-ELISA). Such an assay is used for detecting toxoplasma specific IgM antibody. Class specific anti-human immunoglobulin is absorbed onto a solid phase to "capture" the IgM. Test serum is added and the IgM content is bound to the solid phase. Antigen (disrupted trophozoite) is added and the antigen bound to specific IgM is then detected by an enzyme-conjugated specific antibody. False positive IgM reactions associated with rheumatoid factor, and false
negative results due to an excess of IgG in test sera are reduced in the DS-ELISA system.\textsuperscript{21} By measuring IgM levels in cases of acute toxoplasmosis with known date of onset DS-ELISA reactivity has been shown, on average, to peak at 2 months and to persist for about 6 months.\textsuperscript{20}

Toxoplasma specific IgG and IgA can also be detected using these techniques. Specific IgA antibodies against P30, a major surface protein of \textit{Toxoplasma gondii}, may be useful markers in acute and congenital infections disappearing from the circulation earlier in the course of infection than anti-P30 IgM. The diagnostic value of specific IgA antibodies in AIDS patients requires further evaluation but only one patient of a group of twenty individuals with biopsy-proven toxoplasmosis associated with AIDS had specific IgA demonstrable by ELISA.\textsuperscript{23}

\textbf{Immunosorbent agglutination assay (ISAGA)}

This assay detects human IgM antibodies to toxoplasma by an initial IgM capture using a monoclonal antibody to the CH\textsubscript{2} domain of the human \(\mu\)-chain and subsequent agglutination of whole toxoplasma trophozoites. Routinely used in France for the diagnosis of acute and congenital toxoplasmosis the ISAGA has now been evaluated and introduced into certain laboratories in this country. The ISAGA has been found to be significantly more sensitive than ELISA methods for detecting specific IgM, with comparable specificity.\textsuperscript{24} There is no definitive reference assay with which IgM tests can be compared.

Unlike ELISA reactivity, which rarely remains positive more than 6 months after acute exposure, the ISAGA will detect specific IgM for a longer period of time. In one study reactivity persisted in 80\% of patients for 12 months after initial infection.\textsuperscript{25}

When investigating samples of serum from AIDS patients with possible CNS toxoplasmosis specific IgM should always be looked for. Primary infections are not usual in this group, but some cases of cerebral reactivation may be accompanied by low levels of specific IgM antibodies. ISAGA being more sensitive than DS-ELISA may be the assay of choice in this situation.\textsuperscript{9}

\textbf{Antigen detection tests}

Experimental assays using ELISA and latex agglutination methodology to detect circulating antigen in acute infections of animals and both primary and reactivated infection of humans have been described.\textsuperscript{26} However, the usual duration of parasitaemia following acute exposure is brief, and the diagnostic value of these tests on serum and other samples remains to be proven.

\textbf{Western blotting}

This technique involves transfer of proteins onto a membrane, usually nitrocellulose. By using a class specific anti-human immunoglobulin and detection system it is possible to analyse patterns of toxoplasma antigens on nitrocellulose strips recognised by antibodies in human serum. Detailed investigations of adult sera at various stages of infection have been performed and their antigenic profiles compared.\textsuperscript{26-28} More work is needed to determine whether this technique is of value in the investigation of congenital toxoplasmosis.\textsuperscript{32} To the authors' knowledge little information has been gained by comparing Western blots from AIDS patients with biopsy-proven CNS toxoplasmosis with asymptomatic dye test positive AIDS patients as controls.

Interpretation of Western blots is complicated by naturally occurring antibodies in non-immune sera that are reactive with toxoplasma antigens. The technique is not quantitative and accurate measurement of antigen molecular weights can be a problem.

\textbf{Cerebrospinal fluid (CSF)}

The diagnostic value of CSF sampling in AIDS patients with possible CNS toxoplasmosis is uncertain. Lumbar puncture is often not performed or contraindicated for clinical reasons. When a sample is obtained analysis often reveals a raised protein concentration with or without a lymphocytosis. Dye tests can be performed on specimens of CSF and in one study 23 of 37 (62\%) patients with AIDS and biopsy-proven CNS toxoplasmosis had toxoplasma specific IgG in the CSF compared to none of 11 patients with AIDS alone.\textsuperscript{33} In the 20 cases of toxoplasmosis associated with AIDS described by Holliman\textsuperscript{9} CSF sampling was only performed on three patients. Two patients had specific IgG in the CSF but the result did not contribute to the final diagnosis. Clearly the absence of specific antibody in the CSF does not exclude cerebral infection.

Chemical staining, culture and specific nucleic acid detection can be performed on CSF samples and yield useful information.

\textbf{Histology}

Histological examination of enlarged lymph nodes from immunocompetent patients with acute toxoplasmosis can be useful. Normal tissue architecture is preserved, with follicular hyperplasia and collections of mononuclear cells at the node periphery as characteristic findings.

The use of invasive techniques such as brain biopsy for diagnosing CNS toxoplasmosis in AIDS patients is controversial. The possibility of CNS pathology of other infectious and non-infectious aetiology in dye test positive AIDS cases cannot be ignored, particularly in certain populations of patients. However, the sensitivity and specificity of brain biopsy for diagnosing CNS toxoplasmosis is variable. Areas of well demarcated necrosis with surrounding inflammatory infiltrate, vasculitis, oedema and mild astrocytosis have been reported.\textsuperscript{14} Cysts, when present, are at the necrotic margins. In one study trophozoites were not demonstrable by conventional haematoxylin and eosin staining in more than 50\% of 48 cases.\textsuperscript{14} Immunohistochemical studies improved the sensitivity.\textsuperscript{14} Pre-biopsy treatment will alter the histological appearance as organisation and chronic abscess formation take place. Open excisional biopsy,
where possible, is preferable to a CT guided needle biopsy. Electron microscopy may also be used to demonstrate trophozoites in histological specimens from AIDS patients.

Diagnostic strategies involving brain biopsy can be considered “aggressive” or “conservative”. In the former routine biopsy is performed in all AIDS patients with compatible clinical, serology and CT findings. Alternatively, and more usually, an empirical trial of anti-parasite therapy can be given (rather than brain biopsy) to these patients, and biopsy reserved for cases with negative serology, atypical CT findings, or when therapy fails.

Isolation of the parasite

Toxoplasma gondii can be isolated from biopsy specimens by intraperitoneal inoculation into infection-free mice. Definitive results may not be available for 6–8 weeks. Inoculation of cell lines (such as human embryonic lung) which are then examined by immunofluorescence may produce more rapid results but are less sensitive. Regulations in our laboratory do not permit the injection of samples from AIDS patients into animals, and using a cell culture technique on specimens from two of 20 AIDS patients who underwent brain biopsy the parasite was isolated in neither case despite positive histology and specific nucleic acid detection. Cell culture isolation of toxoplasma from blood of a patient with AIDS has been reported and in the rare cases of pulmonary toxoplasmosis in AIDS inoculation of bronchoalveolar lavage into cell culture has been successful.

The problem with these methods is that they cannot differentiate active infection from the presence of quiescent tissue cysts. The techniques are commonly used in the investigation of foetuses at risk of congenital infection but may be underutilised, when compared with histology, in patients with AIDS. Interpretation of culture results in AIDS patients may be a problem when prophylactic regimes involving antimicrobial agents with activity against Toxoplasma gondii have been used.

Molecular techniques

Recombinant DNA technology has led to great developments in microbiological diagnosis. Specific nucleic acid detection in clinical samples using DNA probes and a hybridisation reaction provides an alternative to culture and serological tests, and has been used successfully for several bacterial, viral and protozoan pathogens. The smaller the quantity of specific DNA in a clinical specimen that can be detected by a hybridisation reaction the greater the sensitivity of the system. When the number of organisms in a specimen is small, sensitivity can be inadequate. Target DNA amplification by the polymerase chain reaction (PCR) dramatically improves the sensitivity of such DNA assays. PCR involves repeated, automated cycles of denaturing double stranded to single stranded DNA, annealing specific oligonucleotide primers to regions either side of the target sequence and extension of DNA synthesis between the primers. This results in a million fold amplification of a target DNA sequence within a few hours. The PCR product can then be detected by agarose gel electrophoresis, transfer of DNA onto membranes (Southern blotting) followed by hybridisation with specific labelled DNA probes, or using new colorimetric methods more suitable for mass screening. Signal amplification using recombinant RNA molecules that function both as specific probe and template for exponential amplification by the enzyme Qβ replicase has been described.

These molecular techniques have been applied in the detection of Toxoplasma gondii. Savva has reported the construction of a genomic DNA library from T gondii but did not reveal any repetitive sequences that would make an ideal DNA probe. Using uncharacterised cloned DNA fragments and dot hybridisation the minimum amount of T gondii detected by these assays corresponded to more than 10⁴ trophozoites which may be too insensitive for infected tissue samples. Recently a PCR assay has been developed that amplifies part of the P30 gene of T gondii DNA. This technique can detect specific DNA in brains and body fluids from mice infected 72 hours previously. The minimum amount of DNA that can be detected is 0.05 pg possibly corresponding to a single organism. Certain clinical situations these assays may have a complementary role to the more conventional diagnostic techniques. Foetal blood and products of conception may be examined to assist in a diagnosis of congenital infection. The PCR can be applied to brain biopsies from AIDS patients but unfortunately the demonstration of T gondii DNA again does not help to distinguish active infection from quiescent cysts. An understanding of toxoplasma stage specific gene expression could be of value in developing techniques to distinguish trophozoite from bradyzoite in the cyst form.

Although PCR has had an immediate and dramatic impact on molecular diagnosis it is not a technique free of problems which may limit its use in the laboratory. The extraordinary sensitivity of the assay may bring into question previous “gold standards” of diagnosis, contamination and inhibitors can complicate matters, and PCR is not essentially a quantitative technique.

Conclusions

Existing laboratory methods for the diagnosis of toxoplasmosis utilise serology, histology, culture and molecular techniques. In the immunosuppressed and the foetus, where an accurate and rapid diagnosis is most critical, a combination of all these methods is often most appropriate. To some extent this reflects the limitations of the individual techniques. Demonstrating exposure to the organism at some time does not pose a problem. However, establishing the exact timing of acute exposure and differentiating a reactivated from latent infection is far more difficult. Addressing these problems is critical to the optimal management of those most at risk from infection. At present...
a diagnosis in an AIDS patient must be made, without delay, on the basis of compatible clinical, radiological and serological findings. This is confirmed by an early response to antitoxoplasma therapy, often within 48 hours and rarely requiring more than 10 days of specific treatment.

43. Savva D. Isolation of a potential DNA probe for Toxoplasma gondii. Microbios 1989;8:105-72.