Title The Diagnosis of Toxoplasmosis in the Immune Compromised Patient

Name Johnson, Julie Dawn

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THE DIAGNOSIS OF TOXOPLASMOSIS
IN THE
IMMUNE COMPROMISED PATIENT

BY

JULIE DAWN JOHNSON

SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF LUTON
Department of Biology and Health Science
Faculty of Science, Technology and Design

UNIVERSITY OF LUTON,
PARK SQUARE,
LUTON,
BEDFORDSHIRE.
LU1 3JU

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ABSTRACT

Human infection with the obligate intracellular protozoan parasite Toxoplasma gondii may be clinically silent in the immune competent person but can cause fatal disease in immune immature, i.e. the foetus, or compromised individuals, such as human immunodeficiency virus infected patients or organ graft recipients. Whilst a number of assays already exist for the laboratory diagnosis of toxoplasmosis, the fact that immune compromised patients present a limited or ablated immune response to primary infection means that many of these are of limited diagnostic value. The aim of the publications presented for this doctorate was to develop, and technically and clinically evaluate assays which would have particular use for the immune compromised patient. The research demonstrates the development of two new serological assays; the immunosorbent agglutination assay for the detection of toxoplasma-specific immunoglobulin-A which was demonstrated to be the investigation of choice for the post-natal diagnosis of congenital toxoplasmosis; the avidity assay which is of value for confirming recently acquired toxoplasma infection in immunoglobulin-M positive pregnant women and for the exclusion of recent onset toxoplasma infection in patients with prolonged immunoglobulin-M responses. It is also of use in the diagnosis of congenital infection. One assay based on detection of parasite deoxyribonucleic acid using the polymerase chain reaction was developed and demonstrated to be optimal in the examination of brain biopsies from acquired immune deficiency syndrome patients with suspected toxoplasma encephalitis, amniotic fluid and foetal blood samples from the potentially infected foetus and cardiac biopsies
from transplant recipients. The research also gives a technical evaluation of two existing assays; the direct agglutination test which proved less sensitive but more specific than the latex agglutination test and demonstrated to be the assay of choice for reducing screening test false positive reactions; the immunosorbent agglutination assay for the detection of toxoplasma-specific immunoglobulin-M which demonstrated to be the investigation of choice for screening women requesting testing during pregnancy, for diagnosis of congenital infection in the foetus and neonate, those suffering from ocular disease and for transplant mismatch cases. The research also clinically evaluates the use of all existing assays for immune compromised patients, concluding with a presentation of a multi-assay approach to diagnosis.
PREFACE

This thesis is submitted, accompanied by the relevant published papers, for the degree of Doctor of Philosophy by publication. The applicant has been actively involved in research into toxoplasmosis since 1989 and consequently also has publications in the subject which are not submitted for this doctorate.

This thesis is therefore presented in sections which will facilitate separation of the publications which are submitted from those that are not.

Sections 1.4 and 1.5 present related research by the applicant presented in patient groups and as diagnostic techniques. The applicant’s references are presented in bold but are not those submitted for the doctorate.

Chapter 3 includes the research presented for the doctorate and all submitted publications are presented in bold.
ACKNOWLEDGEMENTS

All work undertaken in this report was performed between 1989 and 1997 at:-

The Department of Medical Microbiology,
St. George’s Hospital & Medical School,
Blackshaw Road,
London.
SW17 OQT

I would like to thank the staff of The Toxoplasma Reference Unit at St. George’s Hospital for their technical assistance and my father, Mr George Leonard for production of the figures used in this thesis.

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ABBREVIATIONS (Con’t)

TE  Toxoplasma encephalitis

T_H  Helper T cells
CHAPTER ONE

1.0 INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan parasite which was first isolated in 1908 by Nicolle and Manceaux from the tissues of a North African rodent, the gundi (Ctenodactylus gundi). The organism can infect the nucleated cells of virtually all mammals as well as some fish, birds and reptiles. Infection of the immune competent person is often asymptomatic or associated with a mild, self-limiting illness. In contrast, in immune immature, i.e. the foetus, or compromised individuals, such as human immunodeficiency virus (HIV) infected patients or organ graft recipients, this infection can become a severe, life threatening disease.

1.1 EPIDEMIOLOGY

1.1.1 Incidence

The prevalence of antibodies specific to T. gondii within a population is directly proportional to the age of the population, indicating infection is acquired throughout life. In the United Kingdom 0.5-1.0% of the population acquire the infection each year. In addition there are an estimated 1-2 births/1,000 complicated by congenital toxoplasmosis, whilst up to 10% of acquired immune deficiency syndrome (AIDS) patients will suffer an episode of cerebral infection by T. gondii during their life span following diagnosis of their condition.
There are considerable geographic differences in prevalence rates, toxoplasmosis being most common in warm, wet areas with a large cat population where meat is eaten lightly cooked or raw. In the adult population the rate of infection varies considerably, from 10 to 90% in urban areas as close geographically as London and Paris (Gilbert *et al.* 1993; Desmonts and Couvreur 1974).

Sex differences in age distribution of acute disease show a marked predominance of males in the under 20s, thought to reflect more solicitous care of male infants due to their undoubted increased importance over female infants in cultures and/or greater contact with soil. Conversely there is a predominance of infection of women in adult life, thought to reflect either increased risk in the home from cooking and consequently handling and consuming of raw or undercooked meat which may contain infectious tissue cysts (Choi *et al.* 1997) or more intense self investigation of lymphadenopathy (a symptom of infection) in females (Beverley *et al.* 1976).

Many developed countries have noted a decline in the prevalence of the infection, possibly due to an increase in the practice of freezing meat, a process known to reduce the infectious cyst load (Ho-Yen and Joss 1993). It is expected however, that cases of severe toxoplasmosis will increase as the numbers of immune-compromised patients increase with the spread of AIDS (Holliman 1991).

1.1.2 Aetiology and Transmission

*Toxoplasma gondii* reproduces in the intestinal epithelium of the definitive host namely members of the cat family (Figure 1). Cats in the wild become infected soon after weaning by eating infected secondary hosts such as birds or rodents,
**Figure 1. Life Cycle of Toxoplasma gondii**

**DEFINITIVE HOST CAT**

- Tachyzoites transmitted through the placenta during acute infection
- Direct transmission - Human to human infection via direct receipt of tissue, organ or blood transfusion
- Vertical transmission

**INTERMEDIATE HOSTS**

- Ingestion of tissue cysts by predation
- Infection via direct ingestion of sporulated oocyst
- Intermediate host ingests via feed, water, soil, contaminated unwashed vegetables
- Infection via ingestion of tissue cysts by consumption of raw/undercooked meat principally lamb & pork

**Asexual cycle**

- Enteroxys of cat gut
- Favourable external environment 
- Sexual cycle: Sporont non-infectious oocyst passed in cat faeces up to 10 million/day
- Sporocyst
- Sporozoites
- Intermediate host man
- Toxoplasma infection

**Source of figure 1: Original by author of thesis**
hence the prevalence of infection is higher in feral cats. A pregnant cat with a current acute toxoplasma infection may also confer the parasites to her kittens in utero via the placenta. Kittens infected in utero can shed *T. gondii* oocysts after birth (Dubey and Carpenter 1993; Sato et al. 1993).

Oocysts are passed in cat faeces after initial infection for only 1-2 weeks with up to 10 million shed in a day, peak production being 5-8 days post infection. Oocysts remain viable for up to 18 months in soil during favourable conditions and are resistant to most disinfectants (Dubey and Beattie 1988). Although cats can shed *T. gondii* oocysts after reinfection the frequency of repetitive shedding is unknown. Unless sick, little or no faeces will stick to their perianal area and because of grooming faecal matter is not found on the fur of normal cats. Usually cats are not diarrhoeal during the period that they are shedding oocysts and therefore the possibility of transmission of infective parasites to humans, particularly children, by stroking or caring for kittens is minimal.

Human infection may be acquired by ingestion of the oocysts either by direct contact with soil or consumption of unwashed vegetables or contaminated water (Bowie et al. 1997). Alternatively, cats roaming freely around farm areas may defecate in animal feed spreading oocysts and infecting livestock. Ingestion of raw or under-cooked meat containing viable tissue cysts (principally lamb or pork) may result in human infection. Two outbreaks of acute symptomatic toxoplasmosis in Korea have been linked to eating uncooked pork (Choi et al. 1997). Risk factor studies for recent toxoplasma infection in pregnant women undertaken in Naples demonstrated that eating cured pork or raw meat at least
once a month increased the risk of toxoplasma infection threefold (Buffolano et al. 1996) and a similar study in France demonstrated that poor hand hygiene and consumption of undercooked beef were significant risk factors (Baril et al. 1999). The results could be explained by a French tendency to eat undercooked beef and by differences in the animal epidemiology of toxoplasma infection. Ingestion of tachyzoites from an acutely infected animal in contaminated unpasteurised milk may also result in human infection. However, which of these is the main route of infection to humans is not known.

Toxoplasmosis may also be acquired from other humans with acute or latent toxoplasmosis by direct receipt of tissue, organ transplantation or by vertical transmission from an acutely infected mother to her foetus transplacentally. Infection may also be acquired via transfusion of blood or blood products from an acutely infected individual containing tachyzoites of *T. gondii* although this is rare.

1.1.3 Parasitology

Sexual and asexual reproduction occurs in the enterocytes of the definitive host resulting in the excretion of non-infectious oocysts in the faeces and hence into the soil. Depending upon environmental conditions the oocysts sporulate within 3-4 days to form the infective particle, the sporocyst (Figure 2). Ingestion of the sporocyst form of *T. gondii* by a secondary host such as birds, rodents, sheep, pigs or man results in differentiation of sporozoites to tachyzoites which actively invade the cells of the host. Attachment of *T. gondii* to host cells involves a major
Figure 2

Sporocyst of *Toxoplasma gondii*

Scanning Electron Microscopy (Magnification x 16,000)

Source of Figure 2: Produced by the author in collaboration with the Electron Microscopy Department of Medical Microbiology, St George's Hospital London. (Original unpublished photograph).
surface protein, SAG-1 (P30) which is an important parasite ligand that binds to the host cell in the process of *T. gondii* invasion (Mineo and Kasper 1994). The conoid, located at the anterior end of the organism, moves by extension, retraction and rotation and plays a mechanical role in invasion. The rhoptries are elongated, fusiform organelles, located in the anterior half of the parasite extending from the region of the nucleus through the conoid to the anterior plasmalemma. During cell invasion they shorten considerably and within seconds are found as small ovoid saccules at the anterior tip of the parasite which contain enzymes that aid in the penetration of the host cell by causing plasmalemma disruption (Pavesio and Lightman 1996). During the process of invasion an antigen is secreted by the parasite which is incorporated into a parasitophorous vacuolar membrane, which surrounds the intracellular parasite and which differs substantially from endosomal membranes or the membrane of phagolysosomes. The biogenesis and the maintenance of the vacuolar membrane is closely related to the peculiar cellular organization of the parasite (Lingelbach and Joiner 1998; Joiner and Dubremetz 1993).

Tachyzoites appear characteristically crescent shaped, hence the derivation of the name from the Greek *toxon* (arc or bow) and measure 6µm in length and 3µm in width with a single nucleus (Figure 3). Asexual intracellular multiplication of tachyzoites occurs within the parasitophorous vacuole in all nucleated cells and at the same time nucleoside triphosphate hydrolase is synthesized in large amounts and transported through the secretory pathway of the parasite into the vacuolar space in an oxidized and enzymatically inactive form. This nucleoside
FIGURE 3

TACHYZOITES OF *TOXOPLASMA GONDII*
SCANNING ELECTRON MICROSCOPY (Magnification x 10,000)

Source of Figure 3:- Produced by the author in collaboration with the Electron Microscopy Department of Medical Microbiology, St George's Hospital, London.

triphosphate hydrolase is a dense granule protein which, when host cell adenosine tri-phosphate levels fall influences parasite exit from cells leading to disruption and subsequent invasion of adjacent cells (Silverman et al. 1998). A tissue cyst forms in the host principally in brain, heart and skeletal muscle which consists of a host and parasite derived protein cyst wall containing thousands of bradyzoites (Figure 4). Although the bradyzoite appears morphologically identical to the tachyzoite it has a lower metabolic rate. Predation of birds and rodents as secondary hosts by cats completes the life cycle by the ingestion of the tissue cyst form in contaminated tissues. Digestion by proteolytic enzymes in the gut of the predator results in dissolution of the cyst walls which then causes release of the bradyzoite form. This, after entry into intestinal epithelial cells of the host, then differentiates into tachyzoites (Dubey and Beattie 1988; Remington et al. 1994).

*Toxoplasma gondii* strains differ in their virulence in animals, but the extent to which different strains are related is not clear. An analysis of 28 strains from a variety of hosts on five continents found that ten virulent strains had identical genotypes, whereas the nonvirulent strains were moderately polymorphic. This data strongly suggests that the virulent strains of *Toxoplasma gondii* originated from a single lineage which has remained genetically homogeneous despite being globally widespread (Sibley and Boothroyd 1992). A review of studies concerning the genomic drift or polymorphism of *Toxoplasma gondii* including three clones, five zymodemes and 13 schizodemes demonstrated that because mutations occur with some frequency and several allelic configurations were present in isolates grown in the same environment, many of the mutations were not affected by selection pressure.
TISSUE CYST OF *TOXOPLASMA GONDII*
TRANSMISSION ELECTRON MICROSCOPY (Magnification x 6,750)

Source of Figure 4: - Produced by the author in collaboration with the Electron Microscopy Department of Medical Microbiology.
St George's Hospital London.

However, the gametocyte-forming ability was under selection pressure from the host and depended on the development of bradyzoites in tissue cysts. After prolonged multiplication in the tachyzoite stage the gametocyte-forming ability may be lost. No major genomic instability was demonstrated which would justify species or subspecies distinction (Frenkel and Ambroise-Thomas 1997).
1.2 THE CLINICAL PRESENTATION OF TOXOPLASMOSIS

1.2.1 The Immune Competent patient

Following the first exposure to the parasite the period of parasitaemia is limited, usually less than three weeks. Most patients are either asymptomatic or difficult to diagnose. The minority who are symptomatic most commonly present with lymphadenopathy involving both the anterior and posterior cervical nodes (Figure 5). Non-specific symptoms may include fever, lethargy, headache, flu-like illness, myalgia or joint pains lasting from one week to several months. When investigating the clinical syndrome of glandular fever tests for Epstein-Barr virus, cytomegalovirus infection and toxoplasmosis are usually carried out.

In most cases, post-natal toxoplasma infection of the immune competent patient is self limiting and the condition resolves without long term sequelae. Ocular toxoplasmosis is generally considered to be a result of congenital infection. Even in cases where no previous scar is visible in the retina of an adult, it is considered that the inflammation may result from rupture of cysts present in the nerve fibre layer of the retina since birth. However, more recently cases of chorioretinitis in patients with acute acquired toxoplasmosis have been described including a study of families, with no twins, in which three to six siblings had documented retinochoroiditis: many of these patients had immunoglobulin M (IgM) serum antibodies suggesting a recently acquired infection (Pavesio and Lightman 1996). Specific antiparasitic therapy is seldom indicated unless symptoms are protracted or severe.
FIGURE 5

CERVICAL LYMPHADENOPATHY
HISTOLOGY (HAEMATOXALIN AND EOSIN STAIN)
EPITHELEOID GRANULOMA AND GERMINAL CENTRE

Source of Figure 5: Department of Histopathology, St George's Hospital London.
(Original unpublished photograph)
1.2.2 Toxoplasmosis in Pregnancy

The clinical presentation of toxoplasmosis in pregnancy is identical to that in the immune competent patient, although pregnancy itself is a mildly immunosuppressive state. The risk of congenital transmission increases with the gestational age at the onset of maternal infection. This is due to the fact that the parasites find it harder to cross the placenta in early pregnancy but easier later in pregnancy when the placenta is enlarged. However the risk of severe sequelae decreases as the foetus is developing vital organs and limbs early in pregnancy but is fully formed later in pregnancy (Figure 6). There is no evidence to suggest that toxoplasmosis may be transmitted by human breast milk. Limited studies suggest that toxoplasmosis may pass from the immune competent, chronically infected mother (i.e. infected before the onset of pregnancy) to the foetus in 0-6% of pregnancies (Holliman 1994). Approximately 40% of infections acquired during pregnancy are transmitted to the foetus (Carter and Frank 1986), but only 5-10% show severe clinical sequelae at birth.

Due to the asymptomatic nature of the disease, and the severity of any possible sequelae, routine antenatal screening is performed in countries with high seroprevalence rates, e.g. France and Austria. In France antenatal screening is performed during the first trimester of pregnancy using a single test, usually the direct agglutination assay, with monthly retesting of sero-negative women during the second and third trimester in an attempt to detect sero-conversion (Daffos et al. 1988). In a study from Denmark, where prenatal screening is not performed, a neonatal screening programme based on detection of toxoplasma-specific IgM
Figure 6. Transmission of Toxoplasmosis Associated With Pregnancy

Onset of maternal infection

- Pre conception
- 1st Trimester
- 2nd Trimester
- 3rd Trimester

Incidence of foetal infection

- Pre conception: Not known (? rare)
- 1st Trimester: 25%
- 2nd Trimester: 50%
- 3rd Trimester: 65%

Incidence of severe disease at birth

- 1st Trimester: 75%
- 2nd Trimester: 55%
- 3rd Trimester: <5%

Ocular sequelae in later life

- Average risk over entire pregnancy: 65-85%

Average incidence over entire pregnancy (40%)

Source of figure 6: Original by author of thesis
antibodies from phenylketoneuria card eluates has been shown to identify 70-80% of cases of congenital toxoplasmosis (Lebech et al. 1999). However there are inherent difficulties in screening for such a rare condition and the treatment regimes are less than ideal (Greig and Holliman 1999). An antenatal screening programme involving 35,940 pregnant women in Norway revealed that in the first trimester of pregnancy 0.6% of previously uninfected women were falsely identified as positive by the tests used; this percentage increased to 1.3% in the third trimester (Jenum et al. 1998). All putative screening programmes require careful evaluation of the disease, diagnostic tests, management and administrative processes following World Health Organisation recommendations (Wilson and Junger 1968).

In the United Kingdom, the current evidence, namely a calculation of the harm to benefit ratio, does not favour the implementation of routine antenatal screening (Royal College of Obstetrics and Gynaecology 1992). This conclusion was based on the evidence that the benefits of antenatal screening would include a decreased incidence of infants with congenital toxoplasmosis, a reduction in the severity of clinical sequelae seen, improved dissemination of information about the disease to pregnant women and reassurance to those who are shown to be at minimal risk. The disadvantages include an increase in the number of terminations performed due to toxoplasmosis in pregnancy which may or may not be justified, lack of precision in the tests used, diversion of health resources which may be better utilised elsewhere, increased anxiety in the women tested and amelioration of infants that would otherwise have died due to the severity of their infection.
However, more recent serological data from the London area (Gilbert et al. 1993) and South Yorkshire (Walker et al. 1992) have demonstrated evidence of a decline in the prevalence of antibody to *T. gondii* in British pregnant women from 20 to 10%. The decreased incidence of infection may be attributed to several, possibly related factors i.e. improved hygiene avoiding consumption of the parasite from contaminated unwashed vegetables and fruit; the widespread use of frozen meat; the use of intensive farming which separates food animals from cats and better social awareness of risk factors through the distribution of preventative information leaflets advising pregnant women to avoid eating raw or undercooked meat, to wear gloves when gardening, to avoid changing cat litter trays and to wash vegetables and fruit thoroughly before consumption.

The decreased incidence of infection suggests an increase in the population at risk of acquiring an acute infection during pregnancy and it is calculated that as a consequence the number of congenital infections will rise (Ades et al. 1993).

Once a pregnant woman has been found to have an acute infection, investigations may be performed including examination of the amniotic fluid via amniocentesis and sampling of foetal blood by cordocentesis to ascertain whether the foetus is infected with the parasite. Ultrasound examination may be used in an attempt to determine if the foetus is clinically affected. The incidence of foetal loss after cordocentesis is of the order of 2%, depending on the experience of the surgeon. Thus the procedure is only justified when the risk of congenital infection is high and termination of pregnancy is a practical option. Foetal blood and amniotic fluid are assayed by haematological parameters, liver function tests, parasite
isolation and measurement of specific and non-specific antibody levels (Daffos et al. 1988). Parasite isolation from foetal blood and amniotic fluid by tissue culture may provide a rapid but insensitive result.

If acute maternal infection is diagnosed, prophylactic treatment with an anti-protozoal drug (spiramycin) may be given in an attempt to prevent trans-placental infection of the foetus. If cordocentesis, amniocentesis or ultrasound investigations demonstrate foetal infection, the drug therapy may be altered to the more toxic regime of sulphadiazine and pyrimethamine in an attempt to treat the foetus in utero and limit clinical disease. There are no drugs available with reliable activity against the tissue cyst life form of T. gondii and therefore a cure (i.e. eradication of infection) is not currently feasible in this, or any other clinical context.

Due to the acute nature of immunosuppression associated with maternal HIV infection, specifically depletion of Helper T cells which orchestrate the acquired immune response, promoting intracellular killing by macrophages, antibody production by B lymphocytes and clonal expansion of cytotoxic T cells (Fearon and Locksley 1996), it is expected that this will result in increasing numbers of children with congenital toxoplasmosis (Holliman 1994).

1.2.3 Congenital Infection

The classical triad of congenital toxoplasmosis is only seen in 5-10% of cases, comprising intracranial calcification (Figure 7), hydrocephalus (Figure 8) and choroidoretinitis (Eichwald 1960). Non-specific features include intra-uterine growth retardation, hepatosplenomegaly, mental retardation, rash, myocarditis,
CONGENITAL TOXOPLASMOSIS CEREBRAL CALCIFICATION
X-RAY

Source of Figure 7 Radiography Department St George's Hospital London.

CONGENITAL TOXOPLASMOSIS
HYDROCEPHALUS

Source of Figure 8 St George's Hospital London. (Original unpublished photograph)
pneumonia and myositis. Such features are rarely observed in the absence of at least one of the classical features.

Accurate diagnosis is important as conventional treatment is highly toxic to infants. Overwhelming infection may lead to death *in utero* (spontaneous abortion) or during the first weeks of life. Although up to 10% of babies with congenital toxoplasmosis have eye disease apparent at birth, a larger proportion are expected to develop ocular problems in later life. Cohort studies indicate up to 85% of individuals are affected by the third decade of life (Koppe *et al.* 1986).

Cysts may persist throughout life without evoking a host tissue response or may suffer intermittent rupture and cause recurrences of the infection. No definitive conclusions on the origin of reactivation of infection have been reached. Several theories have been proposed including association with rupture of tissue cysts, a hypersensitivity reaction to *T. gondii* antigens released from cysts and the development of hypersensitivity to retinal autoantigens, suggesting that patients become sensitised to autoantigens released from the rods’ outer segments (Pavesio and Lightman 1996). Similarly, the mechanism by which tissue cysts break down is still poorly understood however the possible role of endogenous enzymes, trypsin and pepsin, produced as a product of the inflammatory process and capable of digesting the cyst wall has been proposed (Frenkel and Escajadillo 1987). However, recent incidence studies of patients presenting with ocular disease suggest a significantly lower proportion than the predicted 85% develop ocular disease, but that some racial groups, notably West Africans, have an
enhanced risk of this complication. It is speculated that this may due to strain variation (Gilbert et al. 1995).

1.2.4 Ocular Disease

Ocular disease is classically recognised as one of the clinical conditions associated with *T. gondii* infection. Chorioretinitis (inflammation of the retina) usually results from activation of quiescent cysts established at the time of congenital infection, although post-natally acquired eye disease has been recently reported including a study of families, with no twins, in which three to six siblings had documented retinochoroiditis (Pavesio and Lightman 1996). The degree of visual disturbance depends on the distribution of the lesions within the retina (Figures 9 & 10). If active inflammation occurs, a short course of sulphadiazine, pyrimethamine, folinic acid and anti-inflammatory steroids may be administered. Laser therapy was previously used in an attempt to limit the spread of the disease. By burning around the edge of an active lesion it was hoped to limit progression of retinal damage. This intervention has recently been shown to be of little benefit (Desmettre et al. 1996).

The role of vitreous and anterior puncture is controversial due to its invasive nature however it is routinely performed in Holland for the diagnosis of toxoplasmosis. The Goldman-Whitmer coefficient, which compares levels of specific immunoglobulin from the systemic and intraocular response, is a useful diagnostic tool as is demonstration of intraocular *T. gondii* using the polymerase chain reaction (Minihan et al. 1999). It has been demonstrated, using immunoblotting, that the intraocular *T. gondii* antibody response of patients with
FIGURE 9

OCULAR TOXOPLASMOSIS
FUNDUS HEALED TOXOPLASMA SCAR

FIGURE 10

OCULAR TOXOPLASMOSIS
TOXOPLASMA CHORIORETINITIS
Source of Figures 9 & 10: Department of Ophthalmology, St Thomas's Hospital London. (Original unpublished photograph)
recurrent ocular toxoplasmosis differs from the systemic response. Ocular fluid samples from patients with active ocular toxoplasmosis were shown to contain antibodies that bound a 28 kilodalton (kD) antigen more intensely than did control samples (Klaaren et al. 1998). This finding may have implications for the understanding of the immunopathogenesis of ocular toxoplasmosis.

Demonstration of a characteristic clinical picture with the presence of specific IgG is indicative of infection. Conversely, post-natally acquired ocular disease is more often associated with the typical response seen in the acute stage of infection which includes high level IgM production as expected in the immune competent.

1.2.5 Toxoplasmosis in Organ Graft Recipients

Solid organ transplantation associated toxoplasmosis is most often seen in the context of a transplant mismatch, i.e. a recipient (who has not been previously infected with the parasite) who is given an organ graft from a donor with chronic, quiescent infection (Holliman and Greig 1997). The graft provides the focus of infection and the recipient is unable to restrict the disease process due to the immune suppression therapy given to prevent organ graft rejection.

For example, 50% of heart transplants where a seronegative recipient receives a heart from a seropositive donor will result in a primary *T. gondii* infection reflecting the predilection of tissue cyst formation for muscular tissue (Figure 11). Three to six weeks post transplantation infected recipients develop fever, a depressed mental state and respiratory disease, followed rapidly by death due to
TOXOPLASMOsis IN TRANSPLANTATION

TISSUE CYST OF *T. GONDII* IN HEART MUSCLE

HISTOLOGY HAEMATOXALIN AND EOSIN STAIN (Magnification x 620)

An intact cyst of *T. gondii* within an intact monocyte.
(Note the lack of associated inflammation).

Source of Figure 11 Department of Histopathology St George's Hospital London.

disseminated toxoplasmosis unless specific prophylactic therapy is given (Wreghitt et al. 1989).

Bone marrow and peripheral blood transfusions are unlikely to contain the parasite except in the rare instance of a donor with parasitaemia due to acute, asymptomatic infection at the time of the harvest. Most cases of bone marrow transplant associated disease result from reactivation of the recipient’s pre-existing, previously quiescent, infection due to associated immunosuppressive therapy.

Other types of transplants, such as cornea and bone grafts do not present a significant risk of toxoplasmosis while the risk associated with pancreas or lung transplantation is yet to be established.

1.2.6 Toxoplasmosis and the Acquired Immune Deficiency Syndrome

Toxoplasmosis is the commonest cause of focal cerebral lesions in AIDS patients and usually results from secondary reactivation of infection from previously quiescent cysts in the brain tissue. This is due to profound HIV induced immunosuppression i.e. as seen in HIV patients with a CD4 count below 100 cells/µl. The most common presentation is that of fever, headache, neurological symptoms and rapid deterioration of mental state. Single or, more often, multiple space occupying lesions, which are enhanced with a defined ring appearance after injection of contrast medium, are seen on Computed Tomography (CT) or Magnetic Resonance Imaging (MRI) scans (Laing et al. 1996) (Figure 12). Less commonly the patient may present with cough, dyspnoea and fever associated
FIGURE 12

TOXOPLASMOsis IN AIDS
CEREBRAL TOXOPLASMOsis
COMPUTED TOMOGRAPHY SCAN (arrows indicate "space occupying lesions")

Source of Figure 12: Radiography Department St George's Hospital London
(Original unpublished photograph)
with pulmonary toxoplasmosis or pyrexia and multi-organ failure of disseminated disease.

The differential diagnosis of cerebral toxoplasmosis includes lymphoma, progressive multi-focal leucoencephalopathy, cryptococoma, brain abscess and tuberculoma. HIV infected patients with some preservation of immune function may present with a chronic toxoplasmosis associated illness of prolonged headache, malaise and low grade fever. Diagnosis is often facilitated by a rapid response to therapy comprising high-dose sulphadiazine, pyrimethamine and folinic acid for 6 weeks. Therapy with a reduced dose of the same combination of drugs for the life of the patient is required to prevent relapse.

Early studies on atovaquone (566C80), a hydroxynaphtoquinone drug, demonstrated a significant reduction in the numbers of cysts of T. gondii in the brains of chronically infected mice. The evidence suggested that clinical trials for treatment of toxoplasmosis in AIDS patients using a combination of atovaquone with sulphadiazine or pyrimethamine were justified (Araujo et al. 1993). In a study of a group of 93 patients with cerebral toxoplasmosis with a history of intolerance to, or therapeutic failure of the standard regimen of sulphadiazine and pyrimethamine, atovaquone therapy was shown to lead to clinical improvement or stability in more than one-third (37%) of patients supporting the clinical efficacy of the drug for treatment of toxoplasma encephalitis but not for complete eradication of tissue cysts (Torres et al. 1997).
1.3 TRADITIONAL METHODS FOR THE DIAGNOSIS OF TOXOPLASMOSIS

Due to the diversity of presentation of disease and the latent nature of toxoplasmosis, accurate clinical diagnosis is often difficult or impossible thus leaving laboratory tests as the only diagnostic criteria.

1.3.1 The Immune Response and its use as a Diagnostic Tool

Two general systems of immunity to infectious agents have been selected during evolution; innate, or natural immunity, and acquired or specific immunity. The innate immune systems use proteins encoded in the germ line to identify potential noxious substances (including *T. gondii*) by recognition of carbohydrate structures. Macrophages are also part of the innate immunity and have a receptor for lipopolysaccharide which signals the presence of infection by stimulating the synthesis of chemicals and cytokines, such as interleukin-1 (IL-1), IL-6, IL-12 and tumor necrosis factor, that induce the acute phase response, enhance the microbicidal functions of macrophages and promote the development and growth of helper T cells (*T*<sub>H</sub>). Effectors of the innate immune system also include natural killer cells and antigen presenting cells with complement as the major soluble protein effector. The innate immune response is able to distinguish noxious from innocuous substances but is inflexible and has no "memory", reacting to contemporary (fixed) threats only (Fearon and Locksley 1996).

The acquired immune response, in contrast, is adaptable and establishes a "memory" after initial exposure to provide a rapid response on second exposure. It is not always capable of distinguishing noxious from innocuous substances e.g.
in auto-immune disease but is able to change and develop to meet evolving threats due to its non-fixed nature. B lymphocytes recognise an antigen structure using a protein or carbohydrate target whereas T lymphocytes recognise only protein on cell surfaces such as major histocompatibility complexes (MHC) class I and II. The effectors of the acquired immune response also include macrophages. There are two types of acquired immune response. Type 1 is mediated by macrophages, induces phagocytosis and intracellular killing of bacteria and parasites but also is responsible for auto-immune syndromes and tissue destruction. Septic shock may occur if the type 1 response is over productive. Type 2 does not involve macrophages but is mediated by antibodies, mast cells and eosinophils. T\(_H\) cells exist in two subsets, T\(_H1\) cells promote the type 1 acquired immune response and T\(_H2\) cells promote the type 2 reaction. Development of both subsets can be as a response to signals from the innate immune system. Naïve T\(_H\) cells develop into T\(_H1\) cells following activation of tissue macrophages or natural killer cells or T\(_H2\) cells following antigen presenting cell involvement.

Innate immunity has an instructive role in the acquired immune response determining which antigens the acquired immune system responds to and the nature of that response.

T\(_H\) cells orchestrate the acquired immune response and promote intracellular killing by macrophages, antibody production by B lymphocytes and clonal expansion of cytotoxic T cells. The trigger for production of T\(_H\) cells occurs when bound by complexes (complex = peptides + MHC class II proteins on antigen presenting cells). Innate immunity uses antigen presenting cells or
macrophages to process antigens and display them to the $T_H$ cells or else complement (activated by natural IgM or C-reactive protein) which binds to B cells, improves the B cell to $T_H$ cell interaction leading to improved antibody production (Fearon and Locksley 1996). Antigen recognition at the level of the T cell repertoire can profoundly affect the outcome of disease and the appearance of discrete T cell subsets. It is likely that such mechanisms also underpin genetic susceptibility to diverse other infectious and autoimmune diseases (Locksley et al. 1998).

Cell mediated immunity is the basis of protection of the host from $T. gondii$ infection. However it is of no value in the diagnosis of the disease. Both CD4$^+$ and CD8$^+$ T lymphocytes are principal agents of immunity. It is thought that CD8$^+$ cells play the major role in resistance acting through lysis of $T. gondii$ infected cells or extracellular tachyzoites.

Humoral responses are of secondary importance to cell-mediated immunity in host protection, however they are important in limiting the spread of infection and the degree of disease which develops. Following stimulation, B-cells produce several classes of toxoplasma-specific antibodies. IgM is produced within 2-3 days of infection followed by immunoglobulin G (IgG). Titres of IgG are high initially and then fall to lower levels which remain for the life of the patient. This is thought to be a consequence of stimulation of the immune system due to the continued rupture of tissue cysts releasing tachyzoites into the blood stream. Mucosal immunity has been shown to be initiated principally by the $T. gondii$ SAG1 antigen (formerly known as P30) inducing an early proliferative response in
T lymphocytes in the mesenteric lymph nodes and the spleen. Human intestinal fluid, breast milk and serum have all been shown to contain specific immunoglobulin A (IgA) following toxoplasma infection (Mack and McCleod 1992; Decoster et al. 1988). Comparative studies indicate that maximum levels of IgA are attained later than IgM and typically persist for three months post infection (Gross et al. 1992) (Figure 13).

Immunoglobulin E (IgE) antibodies are produced at the same time as IgM and persistence, dependent upon the sensitivity of the assay used, is typically four months.

Although multiple toxoplasmosis antigens are recognized as targets, antibodies to the 30, 35, 40 and 50 kDa antigens predominate in human sera. The production of antibody, e.g. to \( SAG1 \), which is the major surface protein recognised by serum from acute and chronic disease, enhances cellular based killing of the parasite by activating the classical complement cascade, facilitating lysis of the parasite (Kasper et al. 1986). Other antibodies to surface proteins enhance killing by natural killer and lymphokine activated killer cells (Erbe et al. 1991).

Diagnostic skin tests using purified secretory/excretory antigens have limited clinical applications due to the delay in developing an immune response to the infection but can be used for epidemiological studies (Rougier and Ambrose-Thomas 1985).

Currently, routine laboratory diagnosis of toxoplasmosis relies upon serological methods for detection of circulating antibodies. There are two major types depending upon the antigen source used in each assay. Assays incorporating
Figure 13. The Immune Response In Infection with *Toxoplasma gondii*

Typical immune response in an immune competent patient. Persistence of antibody is dependent upon the sensitivity of the assay used.

Source of figure 13: Original by author of thesis
whole intact organisms as the antigen source include the dye test and fluorescent antibody test. These predominantly detect antibodies produced to surface membrane antigens of the organism and are most sensitive in situations of acute infection.

Assays using disrupted organisms as an antigen source, such as the latex agglutination test and enzyme linked immunosorbent assay, detect antibodies to not only surface membrane antigens but also to antigens derived from the cytoplasm and soluble excreted antigens produced by the organism. These are most sensitive in chronic infection and remain so for a greater period of time. This is thought to be due to the slow continued rupture of tissue cysts and release of tachyzoites producing constant stimulation of the immune system within the host. In general high titres of IgG are seen in acute infection, however, irrespective of the assay employed the magnitude of the antibody responses cannot be used as an indication of the severity of infection due to the fact that protracted high levels of antibody persist in many patients long after the acute stage of the disease.

1.3.1.1 The Dye Test (DT)

The DT of Sabin and Feldman is the accepted classical reference assay for detection of *T. gondii* specific IgG and IgM (Sabin and Feldman 1948). In this test patient serum is mixed with complement and a live antigen source (Figure 14). The presence of specific antibody to *T. gondii* in the presence of complement induces a complement mediated lytic reaction causing cell wall
Figure 14. The Toxoplasma Dye Test

IN A FLAT BOTTOMED MICROTITRE PLATE

Sero-positive patient serum
(possesses specific antibody)
+ Complement source
+ Live trophozoites
(Antigen source)

Seal plate
Mix and incubate at 37°C for 1 hour

Addition of alkaline methylene blue, seal, mix and reincubate at 37°C for 5 Mins

Trophozoites unstained

Sero-negative patient serum
(Does NOT possess specific antibody)
+ Complement source
+ Live trophozoites
(Antigen source)

Seal plate
Mix and incubate at 37°C for 1 hour

Addition of alkaline methylene blue, seal, mix and reincubate at 37°C for 5 Mins

Trophozoites stained

The presence of specific antibody to *T. gondii* in the presence of complement induces cell wall damage to the parasite leaving it unable to retain the vital stain.

Source of figure 14: Original by author of thesis
damage to the parasite leaving it unable to retain the vital stain. This may be seen visually using a microscope.

Sequential dilution of patient serum and production of an appropriate calibration curve facilitates quantitation of antibody titres. Titres are expressed in International Units (IU) as compared with a reference serum. The requirement for a live antigen source relies upon animal inoculation and the assay is therefore restricted to institutions with appropriate animal facilities.

Antibodies reactive in the DT appear within two weeks of infection, reaching a peak within the following two months. Antibody titres decline gradually over many months or years with low IgG titres persisting for life.

1.3.1.2 The Fluorescent Antibody Test (FAT)

The FAT is commercially available and is used widely outside of the United Kingdom but is variable in sensitivity and specificity (Fleck and Kwantes 1980). For quantitative detection of *T. gondii*-specific immunoglobulin killed tachyzoites are fixed on to a slide and incubated with dilutions of the test sample. After washing, fluorescein labelled anti-human IgG or IgM is added and the slides examined for fluorescence using Ultra-Violet microscopy. In using IgM fluorescein conjugates care must be taken to avoid misinterpretation of fluorescence due to non-specific ‘capping’ of the trophozoites. True fluorescence encompasses the whole organism. False positives may also occur with samples containing rheumatoid factor or anti-nuclear factor whilst false negative IgM reactions are observed with samples containing high titres of specific IgG. Fluorescent antibody tests for IgM are not as sensitive as alternative technology.
and therefore results should be confirmed using more sensitive and specific assays.

1.3.1.3 The Latex Agglutination Test (LAT)

The LAT utilizes disrupted organisms as an antigen source fixed to latex beads. The LAT is available commercially as a microtitre tray assay or as a slide agglutination technique. In the microtitre tray format a sample, putatively containing the specific IgG, is added to the latex antigen to produce agglutination after an overnight incubation if positive. The assay may be used quantitatively and has a good correlation with the DT.

The latex slide agglutination assay was introduced in 1991. In this assay polystyrene latex particles are coated with T. gondii soluble antigen and mixed with test sample on a slide. After five minutes rotation the suspension is examined for visible agglutination.

1.3.1.4 The Enzyme Linked Immunosorbent Assay (ELISA)

Numerous direct and indirect ELISAs have been developed for the detection of T. gondii specific IgG, IgM and IgA. The most specific and sensitive of these are direct ELISAs which rely upon coating of microtitre trays with anti-human immunoglobulin and subsequent class specific capture of antibody in a double sandwich technique (Voller et al. 1976; Wreghitt and Morgan-Capner 1990) (Figure 15). Commercial ELISAs for IgG are generally of good quality. However, performance characteristics for IgM ELISAs are extremely variable and as average IgM titres in patients peak at two months and persist for six months,
Figure 15. The Enzyme Linked Immunosorbent Assay For *Toxoplasma gondii*

**Direct method (for antibody detection)**

1. Adsorb class-specific (IgM or IgA) antiglobulin to the solid phase

2. Wash
   - Add unknown sample; (anti-toxoplasma globulin) in the sample is bound by the antiglobulin

3. Wash
   - Add antigen (which attaches to any specific antibody) conjugated to an enzyme-labelled antibody (antigen/conjugate C1E-horse radish peroxidase)

4. Wash
   - Add enzyme substrate (□) (Tetramethy/ benzidine)

   (The product (■) = Amount of specific globulin present in the sample)

5. Stop reaction (2M H$_2$SO$_4$)

6. Measure absorbance using a spectrophotometer

**Indirect method (for antibody detection)**

1. Adsorb the antigen to the solid phase (disrupted trophozoites of *T. gondii*)

2. Wash
   - Add sample; any specific anti-toxoplasma antibody attaches to the antigen

3. Wash
   - Add enzyme-labelled antiglobulin, which attaches to any antibody (rabbit anti-human IgG conjugated to horse-radish peroxidase)

4. Wash
   - Add enzyme substrate (□) (Tetramethylbenzidine)

   (The product (■) = Amount of antibody present in the sample)

5. Stop reaction (2M H$_2$SO$_4$)

6. Measure absorbance using a spectrophotometer

Source of figure 15: Original by author of thesis
they are only of use in acute infection in the immune competent (Payne et al. 1987).

IgM is relatively stable although deterioration may occur on repeated freezing and thawing. Although ELISAs are suitable for processing large numbers of samples they require multiple manipulations. This, and the quest for precise methods for measuring *T. gondii*-specific immunoglobulin, has lead to the development of various automated systems based on ELISA technology.

### 1.3.2 Detection of Parasite

When symptomatic, toxoplasmosis in the immune competent patient most often presents with lymphadenopathy. Differentiation from more serious conditions such as Hodgkins disease, sarcoidosis and leishmaniasis may be attempted by histological examination of tissues, principally lymph nodes. On histology toxoplasmosis typically exhibits follicular hyperplasia and collections of mononuclear cells at the periphery of the node. The use of invasive techniques such as brain biopsy for diagnosing Toxoplasma encephalitis (TE) in AIDS patients is controversial. Areas of well demarcated necrosis with surrounding inflammatory infiltrate, vasculitis, oedema and mild astrocytosis have been reported using histology in brain tissue, with cysts, when present, at the necrotic margins.

Alternatives to detection by physical examination is culture of the organisms from possibly infected tissue and body fluids *in vivo* using either whole animals or cell culture. *T. gondii* can be grown in mice using an intra-peritoneal inoculation technique. Although highly sensitive the method is time consuming, requiring six
to eight weeks, and is not recommended for use with HIV infected tissues due to the risk of operator self-inoculation.

Culture of *T. gondii* can be achieved *in vitro* using inoculation of human embryonic lung (HEL) fibroblasts (tissue culture). Although this is faster producing a result in one to three weeks, it is only 60% as sensitive as animal inoculation (Hughes *et al.* 1986). However, where a rapid result is required, i.e. with foetal blood which may have been taken at 20-22 weeks of gestation, cell culture may still be the method of choice. In such cases a decrease in sensitivity may be acceptable as a rapid result is required should the foetus show signs of infection with *T. gondii* and a termination of pregnancy be necessary. Moreover, when the use of tissue culture was investigated in AIDS patients who underwent brain biopsy, the parasite was not isolated, despite both positive results by histology and Polymerase Chain Reaction (PCR) (Holliman 1991).
1.4 RELATED RESEARCH (Clinical)

The following section, which is presented in patient groups, includes related research on the diagnosis of toxoplasmosis.

1.4.1 The Immune Competent Patient

It has been common practice to perform serological investigations for toxoplasmosis in all patients with glandular fever, who have negative Paul-Bunnell tests results. However, research comparing the incidence of toxoplasmosis in healthy blood donors and in a group of patients with clinically diagnosed glandular fever with negative Paul-Bunnell tests has demonstrated that there is no significant difference in the frequency of acute or chronic toxoplasma infection between these two groups (Johnson and Holliman 1991). The research recommended that Paul-Bunnel negative patients with clinically diagnosed glandular fever are not investigated for toxoplasmosis as a routine. However, these guidelines do not apply to patients at risk of severe sequelae from toxoplasma infection, notably pregnant women, who still require a full assessment. Investigation to exclude more serious causes of lymphadenopathy, particularly lymphoma, should also be performed.

Holliman and Johnson (1991) demonstrated that, depending upon the sensitivity of the assay used, IgM could be demonstrated to persist for approximately six months post infection although rare protracted IgM responses had been reported for up to three years post infection.

Footnote: Applicant’s references in bold are not submitted for this doctorate
In the first and the largest comparison of the LAT against the DT it was demonstrated that the assay suffered from a 1-2% false positive rate due to non-specific IgM (Holliman et al. 1989).

Although false negative LAT reactions are rare, misleading results have been reported in heart transplant recipients (Wreghitt et al 1989). Johnson et al. (1989) determined the performance characteristics of the LAT and concluded that the extreme sensitivity of the assay (99%) made it of optimal use in screening programmes, but that its low specificity (81%) required that serological investigation of the immune compromised by additional assays was necessary.

Related research also included the first and only evaluation of the latex slide agglutination test against the DT. This study demonstrated that the assay was a more rapid and more specific alternative to the LAT while being as sensitive (Dunford and Johnson 1991). However, the predictive value of a negative slide agglutination proved less than the LAT, due to decreased sensitivity when low levels of antibody were present, thus indicating that the assay was inappropriate for the investigation of the immune compromised patient.

One of the first and most successful automated serological assays was the IMX produced by Abbott Diagnostics which has been evaluated in related research against the DT (Hall et al. 1993). However it proved suitable only for investigation of infection in the immune competent due to low sensitivity (false negative reactions) and specificity (false positive reactions).

Footnote: Applicant’s reference in bold are not submitted for this doctorate.
1.4.2 Toxoplasmosis in Pregnancy

Increasing publicity concerning congenital toxoplasmosis has generated a demand for serological assessment by pregnant women concerned of the effects of infection but otherwise in good health. Holliman et al. (1990a) undertook assessments of various assays for this purpose with the conclusion that the LAT is the assay of choice for non-reference laboratories. This research led to the now implemented recommendation, that the dilution of patient serum used in the assay for screening purposes should be 1:16 and that all reactive samples should be subject to further tests or referred to a reference laboratory for definitive confirmation.

Related research involving 141 cases of toxoplasmosis associated with pregnancy demonstrated that 98 (70%) failed to reach a final diagnosis illustrating the need for enhanced education of both clinicians and laboratories dealing with toxoplasmosis in pregnancy (Hartup et al. 1991).

Congenital toxoplasmosis is associated with acute maternal infection acquired during or shortly before the pregnancy. The relative importance of the different sources of toxoplasmosis are not fully established however, recent epidemiological studies have confirmed ingestion of raw and cured meats as a risk factor. Related research by Warnekulasuriya et al. (1998) involving the investigation of 67 ready-to-eat cured meat samples using tissue culture and PCR detected one sample which contained viable T. gondii at a level sufficient to

Footnote: Applicant's references in bold are not submitted for this doctorate.
establish human infection. The research concluded that the health implications of consuming cured meats in pregnancy require careful consideration.

1.4.3 Congenital Infection

Johnson and Holliman (1995) demonstrated that for those at risk of life threatening disease, such as the immune compromised and the neonate, the DT may be performed upon neat serum. The excellent sensitivity and specificity of the assay was maintained even at low serum dilutions unlike most commercial assays.

As a consequence of the poor compliance rate for the diagnosis of toxoplasmosis in pregnancy (Hartup et al. 1991) related research involving pre-natal testing and investigation of infants for toxoplasma infection was undertaken. It was demonstrated that before the study only 30% of cases of possible congenital toxoplasmosis received a substantiated final diagnosis but that with specific intervention by the reference laboratory this was improved to 47% (Hartup et al. 1992).

1.4.4 Ocular Disease

In cases of congenital ocular disease, parasites are thought to reach the retina “\textit{in-utero}”. The immune response mounted is often poor due to the disease being localised to the eye and not systemic. Low IgG titres are produced in the peripheral circulation which rarely rise during an exacerbation of the condition. A study by Holliman et al. (1991a) of 74 cases of retinal disease referred for

Footnote: Applicant's references in bold are not submitted for this doctorate.
confirmation of toxoplasma status, demonstrated that patients with retinal disease have a significantly higher incidence of toxoplasma seropositivity than control groups and that for detection of IgM, immunosorbent agglutination assay (ISAGA) technology gives optimal performance.

McHugh et al. (1997) investigated the application of a tissue cyst antigen ELISA to the diagnosis of ocular toxoplasmosis which indicated that in reactivating disease tissue cyst-specific antibody levels could not be differentiated from those in active disease. The technique was demonstrated to be unsuitable for the diagnosis of ocular toxoplasmosis.

1.4.5 Toxoplasmosis in Organ Graft Recipients

Related research included an audit of the 33 heart transplant cases performed at St. George’s Hospital (Holliman et al. 1991b) which demonstrated that only two seronegative recipients received hearts from seropositive donors. Both were treated with pyrimethamine prophylaxis and remained well.

It was demonstrated that diagnosis of toxoplasmosis in bone marrow transplantation using conventional immunological assays is problematic due to the impaired immune response and thus there is a need for sensitive and rapid diagnostic techniques such as PCR (Johnson et al. 1993; Jurges et al. 1992).

Footnote: Applicant’s references in bold are not submitted for this doctorate.
1.4.6 Toxoplasmosis and the Acquired Immune Deficiency Syndrome

The mechanisms associated with cyst formation and reactivation are not known. Elucidation may lead to eradication of the tissue cyst, therefore erasing the possibility of reactivation. McHugh et al. (1993) developed a viable model for the analysis of the mechanisms of toxoplasma cyst development using HEL fibroblasts as the host cell.

Discrimination between latent T. gondii infection and reactivating toxoplasmosis is difficult using serological techniques. A rapid, accurate diagnosis and treatment significantly reduces morbidity and mortality. McHugh et al. (1997) investigated the hypothesis that antibody recognition of T. gondii tissue cyst-derived antigen (as distinct from tachyzoite-derived antigen) is a good indicator of reactivated disease culminating in the development of an ELISA for antibody to cyst antigens. Eight antigens were consistently recognised by one third of the sera tested but no single antigen was diagnostic of quiescent or active toxoplasmosis. It was concluded that tissue cyst-derived antigens are not a reliable serological marker of reactivating infection.

Related research has demonstrated that PCR is of use in the diagnosis of TE (Holliman et al. 1990b). However, detection of T. gondii nucleic acid in this context does not distinguish active infection from the presence of quiescent cysts.

Footnote: Applicant's references in bold are not submitted for this doctorate.
1.5 RELATED RESEARCH (Diagnostics)

The following section, which is presented as diagnostic techniques, includes related research on the diagnosis of toxoplasmosis.

1.5.1 Detection of Parasite Nucleic Acid
The Polymerase Chain Reaction

A good alternative to serology is the detection of the nucleic acids of a pathogen in clinical specimens. Nucleic acid preparations from infected tissues contain the pathogen’s nucleic acids, as well as those of the host.

Specific sequences in the toxoplasma deoxyribonucleic acid (DNA) may be detected and identified with pathogen-specific DNA or ribonucleic acid (RNA) probes. The complete sequence of the \textit{SAG1} gene encoding the major surface protein antigen of \textit{T. gondii} has been deduced by Burg \textit{et al.} (1988). The gene is 1500 nucleotides in length, polyadenylated and a single copy with no introns.

Preliminary investigations at the University of Reading (Savva 1989) prior to joint development of the \textit{Sag1} PCR assay used original recombinant plasmids derived from a genomic library for \textit{T. gondii} in pUC8, radio-labelled with $^{32}$P using nick translation as DNA probes for the parasite. However the limit of sensitivity of this assay was $\geq 10^4$ parasites ml$^{-1}$ which proved inadequate for clinical use.

Advances in the understanding of the protein structure and function of \textit{T. gondii} facilitated further related research into the identification and sequencing of parts of the parasite genome (Savva \textit{et al.} 1990). This research presented one of the

\textbf{Footnote: Applicant’s references in bold are not submitted for this doctorate.}
earliest applications of PCR to the detection of *T. gondii*, using the *SAG1* gene as an amplification target. The *SAG1* gene is a single copy gene, whereas other workers have developed PCR assays for *T. gondii* based on the B1 gene which is a multi-copy gene repeated 34 times on the genome (Burg *et al.* 1989).

Various reports have since been published describing apparent cross-reactivity of the *SAG1*-gene-base PCR assay for *T. gondii* with *Nocardia asteroides* (McHugh *et al.* 1995a) and *Plasmodium spp* (McHugh *et al.* 1995b). This cross-reactivity has been confirmed with the additional finding of cross-reactivity with *Mycobacterium tuberculosis* (*Holliman et al.* 1996a) but related research also demonstrated that the PCR products generated from these cross reacting species differ in size from the PCR product obtained with *T. gondii* emphasizing the need for strict adherence to published procedures (*Bhalla et al.* 1999). It was evident that stringent hybridisation at 65°C facilitates hybridisation of the *T. gondii* PCR product only and that conditions of lower stringency allows all the cross reacting species PCR products to bind. Electrophoresis in 2% agarose facilitated accurate estimation of product size whilst the cross-reactions were viewed by McHugh *et al.* using 1% agarose.

Footnote: Applicant's references in bold are not submitted for this doctorate.
1.6 RESEARCH AIMS

Whilst the DT remains the “gold standard” assay for the serological diagnosis of toxoplasmosis due to its superior sensitivity and specificity, it is not commercially available. The LAT and FAT have established sensitivity at the expense of specificity. Although they therefore represent excellent screening assays for IgG, they have the potential to yield mis-leading false positive results which complicate diagnosis. Likewise, the IgM ELISA assay is satisfactory for the diagnosis of acute toxoplasmosis in the immune competent but will not detect the low levels of IgM produced in the immune compromised.

1. The aim of the research submitted for this thesis was to develop, and technically and clinically evaluate, assays for the diagnosis of toxoplasmosis which would have particular use for the immune compromised patient. The primary consideration is the fact that this group of patients present a limited or ablated immune response to primary infection.

2. This thesis includes an evaluation and application of the Direct Agglutination Test (DAT) in order to satisfy the specific requirement for increased specificity in the detection of IgG (i.e. excluding false positive reactions). The research submitted aims to ascertain whether the DAT is a viable commercially available alternative to the LAT and FAT that would yield less mis-leading false positive results which currently complicate diagnosis.

3. There is a requirement for an assay with increased sensitivity whilst retaining specificity in the detection of toxoplasma-specific immunoglobulins. The research submitted for this thesis includes the first evaluation of the ISAGA
technique as a potential tool to improve the sensitivity of detection of IgM and investigates the application of the technique for screening pregnant women; for the diagnosis of congenital infection in the foetus and neonate; for the diagnosis of ocular toxoplasmosis; for transplant mismatch cases and the diagnosis of toxoplasma encephalitis.

4. In addition this thesis also includes the development of the ISAGA technique for the detection of IgA in toxoplasmosis which is indicative of recent infection, and an evaluation of its application to the diagnosis of toxoplasmosis in the immune compromised patient. The application of the ISAGA technique is further investigated for the detection of IgA in an attempt to discriminate between asymptomatic latent T. gondii infection and reactivating toxoplasmosis in patients with ocular disease and AIDS.

5. Research submitted includes the development of an assay for the measurement of IgG avidity which is indicative of recent infection, and its application is investigated particularly in the diagnosis of congenital infection. The immune compromised patient is prone to severe life threatening infection with a rapid progression and the requirement for a rapid diagnosis assists in early recognition of disease, prompt treatment and reduced morbidity and mortality. The fact that the immune compromised patient presents a limited or ablated immune response to primary infection prompted the investigation into a direct assay in order to detect the parasite itself rather than rely on the host immune response.
6. This thesis includes research into the development and application of PCR for the diagnosis of toxoplasmosis. The technique is investigated for the diagnosis of toxoplasmosis in the immune compromised patient to provide a rapid alternative to mouse inoculation and tissue culture whilst retaining extreme sensitivity.

7. The research contained in the publications presented in this thesis aims not only to develop and apply new techniques in the varying clinical contexts of immune-compromised patients, but also to compare these with existing technology.
CHAPTER TWO – RESEARCH PRESENTED FOR DOCTORATE

2.0 NEW METHODS FOR THE DIAGNOSIS OF TOXOPLASMOSIS

2.1 SEROLOGICAL DIAGNOSIS

2.1.1 The Direct Agglutination Test

The DAT became commercially available in the United Kingdom in 1988 and although used widely in France as a screening assay, its performance characteristics were not well defined and therefore its suitability as a screening or corroborative assay was not known.

The DAT involves the reaction of specific antibody (IgG) with formalin fixed whole trophozoites to form a visible agglutination pattern (Fulton and Turk 1959). The problem of false positive reactions due to a non-specific IgM as experienced in the LAT is solved by pre-dilution of patient sera in 2-mercaptoethanol which breaks the disulphide bonds binding the IgM molecule. Pre-treatment of the T. gondii trophozoites with the enzyme trypsin is used to increase the sensitivity of the assay by, in part, exposing cytoplasmic antigens lying below the cell wall (Desmonts and Remington 1980). Johnson et al. (1989) published the only clinical evaluation of the DAT as compared to the DT. This study revealed that the DAT has a sensitivity of 96% and a specificity of 98%. Quantitative results with the DAT were not directly comparable to the DT, reflecting the different antibody profiles detected in each assay. The reduced sensitivity of the DAT as

Footnote: Applicant's references in bold are those submitted for doctorate.
compared to the DT limits its use as a screening assay; however, its superior specificity over the LAT demonstrated that it may be used in conjunction with the DT and LAT to confirm false positive results on the LAT especially in the immune compromised patient.

Retrospective serological analysis research includes the only published case of a false-negative DT result (Holliman et al. 1990c). This was recorded in a case of fatal toxoplasmosis associated with cardiac transplantation where the DAT and LAT gave positive titres in the absence of significant reactivity in the DT.

2.1.2 The Immunosorbent Agglutination Assay

The IgM ISAGA became commercially available in the United Kingdom in 1988 (Desmonts et al. 1981). Although the technique was already employed in France for the diagnosis of toxoplasmosis, application of the method into routine diagnosis in conjunction with alternative assays needed to be assessed for all clinical context especially in the immune compromised.

The first clinical evaluation of the ISAGA was undertaken by Duffy et al. (1989). The technique involves an initial IgM capture stage using a monoclonal antibody to the CH domain of the human µ chain followed by the addition of a whole T. gondii trophozoite antigen source to form a visible agglutination pattern (Figure 16). The incidence of false negative reactions, associated with the presence of excess toxoplasma IgG, and false positive results, due to alternative immunoglobulins (known hazards of the conventional ELISA or FAT) is reduced, as is cross-reactivity with IgA and IgE. Coupled with the use of a whole

Footnote: Applicant's references in bold are those submitted for doctorate.
Figure 16.
The Immunosorbent Agglutination Assay for *Toxoplasma gondii*

Wells sensitized with anti-human immunoglobulin
IgM (anti-μ chain)
IgA (anti-α chain)

Add unknown sample and incubate at 37°C for 2 hours. All IgM or IgA in the sample is bound by the antiglobulin

**Wash**
Add whole trophozoite antigen source
(suspension of formalin-treated *T. gondii*
obtained from mouse ascitic fluid in coloured buffer) Incubate at 37°C for 18-24 hours

**Read by eye**

"Agglutination" of antigen in a mat
Sedimentation of antigen to the bottom of the well

**POSITIVE RESULT**
**NEGATIVE RESULT**
trophozoite antigen source the assay produced is highly sensitive and the study demonstrated that it can detect IgM for up to 18 months post infection in the immune competent patient.

Holliman and Johnson (1991) have further proven that as a result of its increased sensitivity i.e. detection of IgM for up to 18 months post infection, the ISAGA may produce a positive result when testing the pregnant woman although initial exposure to *T. gondii* pre-dated conception. This research demonstrated that less sensitive tests such as the ELISA, which only detect IgM on average for 6 months post infection, should therefore be used in such cases as they are indicative of more recent infection.

Investigations by Holliman and Johnson (1989) to define the duration of infection of “at risk” patients, have demonstrated that the ISAGA may be used as a screening assay, with positive sera subjected to further analysis using other assays, such as IgM ELISA and IgG avidity.

Holliman et al. (1991c) and Holliman et al. (1991d) have further concluded that the ISAGA is the assay of choice when investigating infection in the immune compromised individual such as the foetus, neonate and those suffering from ocular disease where IgM production is low or non-existent.

Unlike the DT for the detection of toxoplasma-specific IgG there is no “gold-standard” reference assay for detection of IgM. The IgM ELISA uses a disrupted antigen source, presenting membrane, cytoplasmic and soluble antigens to the serum. The IgM ISAGA incorporates an entire tachyzoite and principally detects

Footnote: Applicant's references in bold are those submitted for doctorate.
antibody directed to membrane antigens. Western blot analysis has shown a considerable variation in quantitative and qualitative antibody response to toxoplasma infection and therefore discordant IgM results may reflect the individuals immune response to specific epitopes (Potasman et al. 1987).

Although a number of techniques have been applied to the detection of IgA in toxoplasma infection including ELISA, ISAGA and Western blotting the relative merits of these tests have yet to be established. Holliman and Johnson (1989) and Patel et al. (1993) undertook research which investigates the development, and provides a clinical evaluation of an IgA ELISA and ISAGA. The IgA ELISA was developed from basic principles using an \( \alpha \)-chain capture technique in a double-sandwich assay. The principle of the test is outlined in Figure 16. The assay was standardised and performance characteristics of reproducibility, sensitivity and specificity determined. The IgA ISAGA was developed from a commercial IgM ISAGA to produce a one well screen assay involving an initial IgA capture stage using a monoclonal antibody to the human \( \alpha \) chain. The assay was standardised and performance characteristics determined. Due to the scarcity of sero-conversion-related series of specimens, patients with lymphadenopathy of known duration were studied to evaluate the assays.

Results from research by Holliman and Johnson (1989) and Patel et al. (1993) have demonstrated that both assays were found to be reproducible and specific tests and that the IgA ISAGA demonstrated enhanced sensitivity. Measurement of IgA in patients with toxoplasma associated lymphadenopathy, of known duration

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with toxoplasma infection, was of limited value due to a significant variation in the IgA level demonstrating that the precise onset of infection could not be determined by IgA estimation in the immune competent. Detection of specific IgA by ISAGA was more sensitive than conventional methods for the diagnosis of congenital toxoplasmosis but it was of limited value in the diagnosis of toxoplasmosis in AIDS patients.

2.1.3 The IgG Avidity Assay

The strength of the interaction between antibody and hapten is defined as ‘affinity’. If the antibody and hapten fit together closely the antibodies bind strongly to the hapten and are referred to as high affinity antibodies. While the term affinity describes the binding of antibody to a monovalent hapten or single antigen determinant, in most practical circumstances it is the interaction of an antiserum (i.e. the serum from an immunized individual) with a multivalent antigen which is of concern. The term employed to express this combined strength of multiple antibody-hapten bonds is ‘avidity’. Studies of the interaction between antibody and antigen have revealed that the strength of binding (affinity) increases as the immune response matures and the duration of infection increases. Thus the combined strength of multiple antibody-hapten bonds also increases. In the diagnosis of toxoplasmosis it is the interaction of patient serum with multivalent antigens which may be measured. *T. gondii* is a protozoan and as such presents external cellular antigens in acute infection to the host immune system.

Footnote: Applicant’s references in bold are those submitted for doctorate.
and soluble excreted antigens, as well as internal cytoplasmic antigens in later infection due to rupture of the parasite.

Measurement of IgG avidity was initially studied in rubella (Thomas and Morgan-Capner 1990; Thomas et al. 1992) and hanta virus (Hedman et al. 1991). The application of this led to the production of IgG avidity assays which detect low-avidity antibodies which predominate in acute infection and high-avidity antibody which is more common in chronic infection (Werbin and Siskind 1972; Hedman et al. 1989).

Holliman et al. (1994) developed the first single dilution step IgG avidity assay for the diagnosis of toxoplasmosis. From evaluations of commercial IgG ELISA assays, one was chosen with maximum specificity of 100% (in order that only the avidity of T. gondii specific antibody were measured) and reduced sensitivity 75%. The assay was then used as a basis for the development of an assay to measure avidity using an elution washing step (Figure 17). A standard dilution of the serum under test was incubated, according to the manufacturer’s instructions, on the solid phase of the ELISA which was an antigen preparation coated onto a microtitre plate.

Based on IgG ELISA technology a “bind and break” method was developed. Instead of washing with a phosphate buffered saline solution after the appropriate incubation period, various concentrations of hydrogen-bond disrupting agents, urea and diethylamine, were used to elute the IgG from the immobilized antigen.

Footnote: Applicant’s references in bold are those submitted for doctorate.
The IgG Avidity Assay for *Toxoplasma gondii*

Indirect ELISA performed in parallel

**Stage 1**
- Plate A (washed with PBS)
- Plate B (washed with elution agent)
- Adsorb the antigen to the solid phase (disrupted trophozoites of *T. gondii*)

**Stage 2**
- Wash
- Add sample; any specific anti-toxoplasma antibody attaches to the antigen

**Stage 3**
- Plate A in phosphate buffered saline (PBS) only
- Plate B➔ in phosphate buffered saline with 6 M urea elution agent (EA)
- Wash

**Stage 4**
- Add enzyme-labelled antiglobulin, which attaches to any antibody (rabbit anti-human IgG conjugated to horse-radish peroxidase)
- Wash

**Stage 5**
- Add enzyme substrate (□) (tetramethylbenzidine)
- (The product (■) = Amount of antibody present in the sample)
- Stop reaction (2M H₂SO₄)

Measure absorbance using a spectrophotometer at 450nm

Calculation of avidity index (AI)

\[
AI = \frac{\text{absorbance after EA wash (plate B)}}{\text{absorbance after PBS wash (plate A)}} \times 100\%
\]
It was demonstrated that 6 M urea used as a washing step after the antigen/antibody binding stage gave optimal differentiation between acute and chronic sera, eluting off low-avidity antibodies whilst retaining high-avidity antibodies. The ELISA was then taken to completion using the specified enzyme and substrate and absorbance readings taken. The IgG avidity index, i.e. the measurement of how much antibody is eluted off of the antigen by the hydrogen bond disrupter was calculated by performing the ELISA in duplicate, one plate incorporating a urea wash step and the other incorporating a wash step with phosphate buffered saline only. The difference between the absorbance readings of the two assays was used to calculate a percentage avidity index.

Using sera from patients with toxoplasma lymphadenopathy of known duration it has been demonstrated (Holliman et al. 1994) that low avidity antibodies persist for up to 3 months post infection and that although a low avidity result is a good indicator of infection of less than 3 months duration a high avidity result is less reliable as an indicator of chronic infection.

2.1.4 The Polymerase Chain Reaction

The SAG1 PCR is a nested assay based on the published sequence for the *T. gondii* SAG1 gene which codes for the major surface protein antigen of *T. gondii*. It consists of 2 rounds of 30 cycles. Specific primers are used, two of which are of the same sense as this sequence and the other two are of the opposite sense resulting in amplification of fragments of *T. gondii* DNA of 914 and 522 base pairs (Figure 18). Using this system a sensitivity of 0.05 picograms of DNA

Footnote: Applicant's references in bold are those submitted for doctorate.  

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Figure 18. Polymerase Chain Reaction for *Toxoplasma gondii* using the SAG 1 Gene

**PCR -ROUND 1**

95°C 1 minute DNA produced

Annealing of specific primers

74°C 3 minutes Extension using Taq polymerase

Repeat 30 times

Production of 914 bp fragments

**PCR -ROUND 2**

95°C 1 minute DNA produced

Annealing of specific "nested primers"

74°C 3 minutes Extension using Taq Polymerase

Repeat 30 TIMES

Production of 522 bp fragments

Gel electrophoresis
(approximately one trophozoite) could be obtained (Johnson et al. 1990). This research presented one of the earliest applications of PCR to the detection of T. gondii. Initial studies involved an evaluation using various mouse and human tissues and body fluids demonstrating the increased sensitivity of the assay over conventional murine inoculation and tissue culture. In most cases PCR complemented and in some cases offered a better alternative to conventional diagnosis and the assay was subsequently successfully used in a routine clinical laboratory for the diagnosis of the disease as illustrated in several of the case reports to be presented in this thesis. The Sagi is a single copy gene, whereas other workers have developed PCR assays for T. gondii based on the B1 gene (Burg et al. 1989) which is a multi-copy gene repeated 34 times on the genome. The sensitivity of a PCR assay based on a repetitive gene, such as the B1 should be greater than that of an assay using a single copy gene target, such as the SAGI, assuming that the efficiency of amplification and number of PCR cycles employed are similar. However, these two assays have not been evaluated simultaneously on human clinical samples. The only published comparison (Wastling et al. 1993) utilized efferent lymph and peripheral blood collected from sheep experimentally infected with T. gondii strain S48. When detection of the parasite directly by culture in whole animals (the gold standard) was compared to the B1 and SAGI PCR, the B1 proved to reflect results that were closer to the gold standard than the SAGI PCR (Wastling et al. 1993). This could be explained by the fact that the B1 gene has multiple short DNA fragments on the genome which

Footnote: Applicant's references in bold are those submitted for doctorate.
provide a better target for PCR than the $SAG1$ which is a longer single copy gene and therefore the former assay is more sensitive. However, where the danger of cross-contamination is greater, the superior sensitivity of B1 PCR dictates that greater stringency in the laboratory is required to avoid false positive results.

Footnote: Applicant’s references in bold are those submitted for doctorate.
CHAPTER THREE – RESEARCH PRESENTED FOR DOCTORATE

3. CLINICAL APPLICATION OF METHODOLOGY

3.1 THE IMMUNE COMPETENT PATIENT

Increasing IgG titres are rarely seen in the immune competent due to the delay in clinical diagnosis and the rapidity of onset of the immune response. More recently acquired infection may be demonstrated using a relatively insensitive ELISA assay. The persistence of specific IgG also makes diagnosis based on IgG alone unreliable. Lymph node biopsy may be performed to exclude more serious conditions, including lymphoma, although demonstration of the parasite does not differentiate between quiescent and active disease.

Holliman et al. (1996b) have proven that IgG avidity is of value in the diagnosis of the immune competent, a low avidity demonstrating infection of less than three months duration. This work has been the only publication to demonstrate that the primary immune response does not usually produce a prolonged positive result with this assay and provided evidence that IgA is of value although prolonged responses after the acute stage of infection do occur.

3.2 TOXOPLASMOsis IN PREGNANCY

If acute toxoplasmosis is diagnosed in the pre-conception period deferment of pregnancy for six months or serial IgM tests to document loss of IgM is generally advised when the risk of parasitaemia is likely to be negligible or lost altogether.

Duffy et al. (1989) have demonstrated that the duration of IgM production

Footnote: Applicant's references in bold are those submitted for doctorate.
exceeds the persistence of parasitaemia and that this is therefore a very conservative approach. This research emphasized the fact that individual cases require assessment on their own merits.

When testing antenatal patients, duration of maternal infection has a significant impact upon treatment and sequelae of disease. The most severe clinical sequelae are seen when infection is acquired during the first trimester of pregnancy, in which case foetal blood sampling or termination may be justified. Holliman et al. (1994) have shown that the IgG avidity assay has a complementary role in the accurate estimation of duration of infection. This research also uniquely demonstrated that an unknown percentage of patients also present with a prolonged IgM response which compromises the estimation of duration of infection and that maturation of IgG avidity is the investigation of choice for such cases. Thirty seven clinical cases where IgM response was known to have persisted for 6-24 months have been considered by Holliman et al. (1996b). Only one case presented with a low avidity as opposed to seven who demonstrated the presence of toxoplasma specific IgA.

The investigation of toxoplasmosis in pregnancy falls into two basic categories depending upon the existence or absence of screening programmes. Optimally, seroconversion in serial samples using a reliable IgG assay would be the method of choice. Women found to lack specific IgG should be offered health education and retested. In the absence of immune suppression, women with chronic latent infection can be reassured. Patients with recently acquired infection

Footnote: Applicant’s references in bold are those submitted for doctorate.
3.3 CONGENITAL INFECTION

A case report demonstrating the difficulties in the diagnosis of congenital toxoplasmosis by cordocentesis using several of the diagnostic techniques outlined is described by Holliman et al. (1991d). This is the only published case of a false positive ISAGA in this clinical context. In this a 28 year-old woman presented for ante-natal booking at 17 weeks gestation, of apparently good health but worried about toxoplasmosis. After appropriate counselling, serological testing revealed an acute infection. Foreign travel eight weeks and a mild flu-like illness six weeks before conception was demonstrated. Ultrasound studies showed a normal foetus and the pregnancy continued with prophylactic spiramycin 3g daily. At 23 weeks ultrasound was repeated and cordocentesis undertaken.

The purity of the foetal blood was confirmed by a Kleihauer test and serological analysis revealed the presence of IgG using the DT, LAT and DA, the IgM by ELISA was negative but the IgM by ISAGA was positive. All animal inoculation studies were negative for culture and PCR revealed a negative result for amniotic fluid although the foetal blood was not tested. At birth and 3, 28 and 30 weeks post delivery IgM was absent from the child and IgG levels declined to negative. This finding of an ISAGA reactive blood sample from an uninfected child may

Footnote: Applicant’s references in bold are those submitted for doctorate.
have been a false positive reaction, although the Kleihauer test demonstrated the purity of the foetal blood indicating the absence of placental trauma. The conclusion from this research was that to achieve optimum care of the foetus a multi-disciplinary approach is advocated (*Holliman et al. 1991d*).

For serological diagnosis of the neonate, when taking blood by venepuncture is technically difficult, collection of whole blood onto filter paper after a heel prick requires minimal surgical skill and can be performed by nursing staff visiting the child’s home. Studies have shown that eluates from these filter papers give a sensitivity of 10IU by the DT, 53 Enzyme International Units by the IgM ELISA and that IgM ISAGA findings using eluate or sera were comparable (Patel and Holliman 1994).

**Holliman and Johnson (1989)** reviewed a total of 20 cases of suspected congenital toxoplasmosis investigated in the post natal period. Ten children were found not to be infected and the diagnosis of congenital toxoplasmosis was confirmed in 10 infants by the persistence of specific antibody at the age of 12 months, with or without clinical signs of infection. Of these 10 cases of congenital infection, three had toxoplasma specific IgM detected by ELISA and ISAGA all of which demonstrated one or more of the classic triad of symptoms of the disease, retinochoroiditis, cerebral calcification or hydrocephalus. A further three had toxoplasma specific IgM detected by ISAGA alone two of which were asymptomatic and one who had developed macular scars. The remaining four had no detectable IgM two of which were asymptomatic, one who demonstrated

*Footnote: Applicant’s references in bold are those submitted for doctorate.*
dilatation of ventricles and one who demonstrated hydrocephalus. This highlighted the need for improved diagnostic methods for the diagnosis of congenital toxoplasmosis and lead to related research into the value of detection of IgA in such cases. **Patel et al. (1993)** have demonstrated the detection of IgA to be a more sensitive indicator of congenital toxoplasmosis than detection of IgM. In a study of 17 babies with congenital toxoplasmosis, 12 (60%) were correctly identified by IgA ISAGA as compared to seven (30%) by IgM ISAGA. In the absence of specific IgA, IgM or clinical symptoms persistence of specific IgG beyond one year of age confirms the diagnosis of congenital toxoplasmosis.

The work has also demonstrated that diagnosis by ISAGA may allow specific therapy to be given during the first year of the child’s life, potentially reducing sequelae of infection (**Holliman and Johnson 1989; Patel et al. 1993**). At the time of developing the IgA ISAGA technique there were no commercially available alternatives and thus this assay was unique in itself.

Evidence is also provided (**Holliman et al. 1994**), demonstrating that serial measurement of IgG avidity assists in the discrimination between active production of antibody with congenital toxoplasmosis and passively acquired antibody of maternal origin associated with the uninfected child. This has not been demonstrated by any other workers.

An audit was undertaken by **Hartup et al. (1997)** to determine case compliance with prenatal testing and investigation of infants for toxoplasma infection. From this it was shown that the status of the child was established in only 30% of cases.

**Footnote: Applicant’s references in bold are those submitted for doctorate.**
when acute maternal infection was detected. This demonstrated that toxoplasma infection is grossly underdiagnosed in the neonate. The research further demonstrated that with continuous follow-up in the form of informative and reminder communication to the paediatrician in charge, the status of the child was established in 45% of cases over a three year period, but that this increase seen in case compliance was lost once the stimulus was withdrawn. The most frequently established cause of case non-compliance was found to be clinician/local laboratory error as many children were erroneously discharged on the basis of a normal clinical examination.

3.4 TOXOPLASMOSIS IN ORGAN GRAFT RECIPIENTS

Primary toxoplasma infection acquired via the transplanted heart can usually be demonstrated by serological or histological methods. The infected recipient usually has detectable specific IgM and rising titres of IgG, but discordant findings have been demonstrated.

A unique case report recording a false-negative dye-test result in a case of fatal toxoplasmosis associated with cardiac transplantation when other assays showed significantly rising titres is described by Holliman et al. (1990c). A 58-year-old sero-negative recipient had received a seropositive heart and was not treated prophylactically for toxoplasmosis due to the demonstration of stable DT titres. He became pyrexial after 18 days with evidence of pulmonary collapse on chest X-ray. All cultures proved negative and the patient was discharged. However, 54 days post transplant he died suddenly at home. Retrospective serological analysis

Footnote: Applicant’s references in bold are those submitted for doctorate.
revealed that although the dye test titre remained stable at 4 IU the LAT and DA showed significantly rising titres and that both IgM ELISA and IgM ISAGA were positive by 30 days post transplant. The fact that the DT remained stable whilst the other tests rose in titre may be due to the different antigenic profiles presented by each assay or to failure of the patient’s immune system to produce complement fixing antibody.

An important outcome of this case history is that careful consideration of ALL serological and histological results needs to be undertaken in cases of suspected toxoplasmosis of the immune compromised. All cardiac transplant mis-matches are now given prophylactic pyrimethamine during the immediate post-operative period at St. George’s Hospital.

Holliman et al. (1992) assessed the conventional diagnostic technique of histology against PCR using a series of 15 cardiac biopsy samples taken from seven transplant recipients around the time of primary toxoplasma infection. T. gondii was detected in two of the seven cases by histology and in five of the seven cases using PCR, demonstrating the superior sensitivity of PCR and the ability of this test to produce results in a clinically relevant time period making this the assay of choice in heart transplantation. PCR makes possible the detection of very small numbers of pathogens in clinical specimens, providing a rapid alternative to mouse inoculation and tissue culture.

Footnote: Applicant’s references in bold are those submitted for doctorate.
3.5 TOXOPLASMOSIS AND AIDS

Determination of base line toxoplasma specific IgG titres is essential due to the recrudescent nature of the disease commonly resulting in cerebral involvement. Observation of clinically diagnostic features may be confirmed by a therapeutic trial of high dose antiparasitic agents. It is rare for IgG titres to rise during reactivation and low level IgM response may only be detected by ISAGA technology.

This is illustrated by Holliman et al. (1991c) in the first case report of PCR used in this clinical context. The report describes a 53 year-old-male homosexual who first became unwell in January 1988. Eight months after his initial presentation the patient developed polyneuropathy and a further six months later he noted a change in his gait with evidence of muscular weakness due to a left-sided motor neurone lesion.

A CT scan revealed a typical presentation of toxoplasmosis and treatment with pyrimethamine, sulphadiazine and folinic acid began. The patient suffered grand mal seizures and a brain biopsy was performed. The presence of *T. gondii* DNA was demonstrated by PCR in the brain biopsy and in a peripheral blood sample taken at the same time. The patient developed a generalised erythematous skin rash due to the sulphonamide and was switched to clindamycin and subsequently dapsone.

At the time of initial presentation and at the time of the brain biopsy both the DT and LAT titres remained static and IgM by ELISA was negative. However IgM

Footnote: Applicant's references in bold are those submitted for doctorate.
activity was detected by ISAGA at the time of relapse.

Research by Patel et al. (1993) into the value of IgA in the diagnosis of toxoplasmosis in AIDS demonstrated that it is was of little value.

Footnote: Applicant's references in bold are those submitted for doctorate.
CHAPTER FOUR

4. CONCLUSIONS

Toxoplasmosis represents a life-threatening opportunistic infection to the immune compromised patient and the aim of the research undertaken during this thesis was to develop and evaluate technically and clinically, assays for the diagnosis of toxoplasmosis which would have particular use for immune compromised patients.

This aim has been achieved with the following conclusions:-

1. The research submitted demonstrates the development of two new serological assays and one assay based on detection of parasite DNA. It also gives a technical evaluation of two existing assays and clinically evaluates the use of all existing assays for immune compromised patients.

2. The Direct Agglutination Test is established as the assay of choice when alternative screening tests produce false positive reactions due to non-specific IgM. It is a viable, commercially available alternative to existing techniques for the detection of toxoplasma-specific IgG and its high specificity makes it a complimentary assay which will not yield false positive results (Johnson et al. 1989).

3. The Immunosorbent Agglutination Assay for the detection of toxoplasma specific IgM is demonstrated to be highly sensitive. It is demonstrated to be the investigation of choice for; non-prescriptive screening of pregnant women but not for the diagnosis of acute toxoplasmosis in pregnancy (Duffy et al. 1989); for diagnosis of congenital infection in the foetus and neonate
(Holliman and Johnson 1989; Holliman et al. 1991d) and those suffering from ocular disease and for transplant mismatch cases (Holliman et al. 1990c). It is of limited use in the diagnosis of TE. It should be noted that there are statistical and practical limitations of studies performed on small sample sizes such as illustrated in Holliman and Johnson 1989, however as toxoplasmosis is a relatively rare condition in the United Kingdom specimens from infected individuals may take years to acquire.

4. The Immunosorbent Agglutination Assay for the detection of toxoplasma specific IgA is the investigation of choice for the post-natal diagnosis of congenital toxoplasmosis, the case compliance rate of which was demonstrated to be sub-optimal (Patel et al. 1993; Hartup et al. 1997). It is also of value for the diagnosis of cerebral toxoplasmosis associated with primary infection in AIDS patients. IgA is not of value for the exclusion of recent onset toxoplasma infection in patients with prolonged IgM response or for diagnosis of ocular disease (Holliman et al. 1996b).

5. The IgG Avidity Assay is of value for confirming recently acquired toxoplasma infection in IgM positive pregnant women and for the exclusion of recent onset toxoplasma infection in patients with prolonged IgM response (Holliman et al. 1996b). It is also of use in the diagnosis of congenital infection (Holliman et al. 1994).

6. The Polymerase Chain Reaction is optimal in the examination of brain biopsies from AIDS patients with suspected TE (Holliman et al. 1991c), amniotic fluid and foetal blood samples from the potentially congenitally
infected foetus and cardiac biopsies (Holliman et al. 1990c; Holliman et al. 1992) and bone marrow from transplantation. PCR should only be used where the integrity of the specimen can be guaranteed due to its extreme sensitivity.

7. Diagnosis of Toxoplasmosis Using a Multi-Assay Approach is demonstrated in Table 1.
Table 1. Diagnosis Of Toxoplasma Infection

<table>
<thead>
<tr>
<th>Immune competent patient lymphadenopathy</th>
<th>Immune competent patient</th>
<th>Toxoplasmosis in pregnancy</th>
<th>Pre-conception screening and antenatal screening</th>
<th>Acute infection in pregnancy</th>
<th>Congenital infection</th>
<th>Foetal and neonatal assessment</th>
<th>Ocular disease</th>
<th>Organ graft recipients</th>
<th>Pre-transplant screening (donor &amp; recipient)</th>
<th>Infected heart / lung / liver recipient</th>
<th>Infected bone marrow recipient</th>
<th>Toxoplasmosis and AIDS</th>
<th>HIV positive individual</th>
<th>Cerebral disease and AIDS</th>
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= New methods developed as a part of this report.

X = Methods inappropriate for use in diagnosis.

✓ = Methods appropriate for diagnosis (often used in combination)

Source of table 1: Original by author of thesis.
REFERENCES


Footnote – Bold & Highlighted = Applicant’s references submitted for doctorate.

Bold only = Additional references of applicant.


Footnote - Bold & Highlighted = Applicant's references submitted for doctorate.
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Footnotea- Bold & Highlighted = Applicant's references submitted for doctorate. Bold only = Additional references of applicant.


Footnote – Bold & Highlighted = Applicant’s references submitted for doctorate.
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Footnotes- Bold & Highlighted = Applicant’s references submitted for doctorate. Bold only = Additional references of applicant.


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**Footnote – Bold & Highlighted** = Applicant’s references submitted for doctorate.
**Bold only** = Additional references of applicant.


LIST OF WORKS SUBMITTED FOR DOCTORATE


**APPLICANT STATEMENT**

The above named publications form the work upon which the candidature is based.

All work undertaken was performed between 1989-1997.

All work undertaken was performed in:-

The Department of Medical Microbiology,
St. George’s Hospital & Medical School,
Blackshaw Road,
London
SW17 0QT