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**Development of a Monoclonal-Antibody Based
Antigen Detection Enzyme Linked Immunosorbent
Assay (ELISA) for the Diagnosis of Human
Toxoplasmosis**

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A thesis submitted to the Faculty of Graduate Studies and Research in partial
fulfilment of the requirements of the degree of Master of Science.

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ABSTRACT

Although several commercial serological kits exist for *Toxoplasma* serodiagnosis, the unambiguous diagnosis of many clinically important *Toxoplasma* infections remains problematic. This is particularly true in establishing the timing of infection in pregnant women and in demonstrating reactivation of disease in immunocompromised hosts. The wide tissue tropism and distinct life-cycle stages of toxoplasmosis raise the possibility that the detection of circulating tachyzoite antigens may be of use in these situations. We have developed a series of antigen capture Enzyme Linked Immunosorbent Assays (ELISAs) using a panel of novel monoclonal antibodies (mAbs) directed against *T. gondii* tachyzoite antigens. Using a pool of these mAbs to capture whole tachyzoite lysate antigen in 'spiked' negative serum, the detection limit of our ELISA was 1-2 μ g/ml of protein. The sensitivity of this ELISA was 52% (n=412). We postulated that our low sensitivity was mainly due to circulating immune complexes. This was confirmed by the disappearance of 'spiked' tachyzoite lysate antigen in antibody positive samples followed by the reappearance of antigen upon 12% trichloroacetic acid (TCA) treatment. Furthermore, the use of 12% TCA significantly increased antigen detection (p=0.00001). Preliminary results suggested that assay sensitivity was 96% (n=254) while assay specificity was 97% (n=253). Early reports suggest that *Toxoplasma* antigen levels in serum are transient. The magnitude and kinetics of antigenemia with the specific *Toxoplasma* products recognized by our panel of mAbs remain to be determined. This optimized assay can now be used to test sera from otherwise healthy and immunocompromised subjects to determine its clinical utility.

RÉSUMÉ

Plusieurs tests commerciaux existent pour le sérodiagnostic de la toxoplasmose. Mais, l'ambiguïté du diagnostic de certaines infections cliniquement importantes restent problématiques, en particulier chez les femmes enceintes et les hôtes immunodéficients. La diversité du tropisme tissulaire et les différents stages du cycle du parasite évoquent la possibilité que la détection des antigènes tachyzoïtes (AT) peut être utiles dans ces situations. Nous avons développé des séries de test immunoenzymatique (ELISA) à détection d'antigènes utilisant des nouveaux anticorps monoclonaux (aCm) dirigés contre les AT du parasite. Utilisant tous ces aCm pour détecter les "ajouts" de lysats entiers d'AT dans les sérums non-infectés, la limite de détection de l'ELISA a été de 1-2µg/ml de protéine. La sensibilité de l'ELISA a été de 52% (n=412). Nous avons postulé que notre seuil de sensibilité est principalement dû à la circulation des complexes immuns. Ceci a été confirmé par la disparition des "ajouts" de lysats d'AT au niveau des échantillons positifs suivi par une réapparition des AT avec un traitement de 12% d'acide trichloroacétique (ATC). De plus, l'utilisation de 12% d'ATC a augmenté significativement la détection d'antigène ($p=0.00001$). Les résultats préliminaires ont suggéré que la sensibilité de l'essai était de 96% (n=254) et la spécificité a été de 97% (n=253). Les rapports suggèrent que les niveaux d'antigène de *T. gondii* dans le sérum sont éphémères. La grandeur et la cinétique de l'antigénémie avec les produits spécifiques reconnus par notre liste d'aCm restent à être déterminer. Cet essai peut être maintenant utiliser pour tester les sérums provenant aussi bien de patients sains que de patients immunodéficients pour déterminer leur utilité clinique.

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INTRODUCTION & RATIONALE

INTRODUCTION & RATIONALE

Toxoplasma gondii is one of the principal protozoan parasites of man (1). *T. gondii* is classified as a heteroxenous or two-host coccidian parasite of cats (primary host) and mammals (secondary host), forming tissue cysts. It is grouped in the subfamily *Toxoplasmatinae*, family *Sarcocystidae*, suborder *Eimeriorinia*, order *Eucoccidiida*, subclass *Coccidiasina*, and class *Sporozoasida*, within the protozoan phylum *Apicomplexa* (1, 2). *Toxoplasma gondii* infects a wide range of vertebrate hosts. In humans, toxoplasmosis may be acute or chronic, symptomatic or asymptomatic. Typically, infection is asymptomatic in immunocompetent hosts. When symptoms develop, they are usually short-lived and self-limited in most individuals (1, 2, 3). Acute infection poses greatest hazard to the infant *in utero* and to the immunocompromised patient (1, 2, 3). Human toxoplasmosis is acquired primarily through the ingestion of raw or undercooked infected meat products containing tissue cysts or from contact with soil contaminated with cat feces (1, 2, 3). The diagnostic tests currently available for the detection of toxoplasmosis rely solely on antibody detection and are often inadequate for the diagnosis of congenital infection (e.g.: establishing the timing of infection in pregnant women) and reactivated disease in the immunocompromised host. The distinct life-cycle stages and broad tissue tropism of *T. gondii* raises the possibility that the detection of circulating tachyzoite antigens may be of use in these situations (4, 5). To date, experience with antigen detection techniques in human *Toxoplasma* infections is limited (4, 5). Thus, the aim of this study was to design a highly sensitive and specific *Toxoplasma* antigen detection assay that could significantly advance the current diagnostic strategies.

CHAPTER 1

LITERATURE REVIEW

1.1 – HISTORICAL BACKGROUND

Toxoplasma gondii was originally described in animals by Nicolle and Manceaux (in a desert rodent – the gondi) (6) and by Splendore (in rabbits) (7) in 1908 and was given its name the following year (6). Wolf, Cowen and Paige first observed human congenital infection between 1937 and 1939 (8). In 1939, acquired infection was described by Sabin in children (9) and by Pinkerton and Weinman in adults (10). In 1948, Sabin and Feldman introduced the first serological test that led to the recognition of asymptomatic infection in human and animal populations throughout the world (11). Remington and collaborators would later create IgG and IgM antibody detection ELISAs in the 1970s and 1980s. These tools have become the standard diagnostic tests worldwide (12, 13, 14). Of great importance, Frenkel, Dubey and Miller described the life cycle of *T. gondii*, its coccidian nature and the role of cats between 1969 and 1970 (15, 16).

1.2 – LIFE CYCLE

1.2.1 – General Introduction

The definitive hosts of *T. gondii* are members of the feline family. There are two distinct “loops” in the life cycle of *T. gondii* (15). For example, in the domestic cat, the organism undergoes the complete life cycle, including both an enteroepithelial loop and an extraintestinal loop (15). The enteroepithelial cycle of development is similar to that of other coccidians such as *Cystoisospora*, *Hammondia*, *Besnoitia*, *Sarcocystis*, and *Frenkelia* (1, 3). In other mammalian and avian hosts, the organism exhibits only the extraintestinal phase of development. The enteroepithelial loop

results in the production of oocysts, whereas in the extraintestinal loop, only tachyzoites and tissue cysts are present (15).

Definitive and secondary hosts can be infected by eating meat containing **bradyzoites** or **tachyzoites** or through the ingestion of **sporulated oocysts**. Sporozoites released from the cysts infect a wide variety of tissues and rapidly undergo endodyogeny to form **tachyzoites**. These can infect other tissues in the body, such as muscles, fibroblasts, the liver and nerves. Asexual reproduction in these tissues is much slower than at the original site and the parasite develops large cystlike accumulations of tachyzoites that are now called **bradyzoites**. The cyst itself is called a zoitocyst, or simply a **tissue cyst**.

1.2.2 – Oocyst Production

After a feline (e.g.: domestic or wild cat) ingests either tissue cysts or oocysts, *T. gondii* sporozoites are released and invade the epithelial cells of the small intestine where they enter the enteroepithelial loop. The organism undergoes sequential stages of development and multiplication, resulting in the formation of oocysts. Gametocytes are found throughout the small intestine. Each male gametocyte produces approximately 12 microgametes, which escape and fertilize macrogametes. Following fertilization, an oocystic wall surrounds the zygote and the oocysts are discharged into the lumen, exiting the host with the feces. Sporogony occurs outside the body and requires 2-21 days (15). The zygote further develops by dividing into two sporoblasts. Each sporoblast develops a wall and thereby becomes a sporocyst.

Within each sporocyst there are two further divisions, producing four sporozoites. Therefore, each oocyst contains eight sporozoites. The prepatent period to oocyst production varies between 3-24 days depending on the form of the organism ingested (15, 16). The sporulated oocysts are infectious and an infected cat can excrete millions of oocysts each day for approximately 2-3 weeks. Ovoid in shape and 10-12µm in diameter, oocysts have been found only in members of the feline family (15, 16).

1.2.3 – Tachyzoite Production

The extraintestinal cycle begins after ingestion of oocysts by either felines or secondary hosts, in which viable organisms (sporozoites) are released and invade the epithelial cells of the intestine (1, 3). In felines, some of the sporozoites enter epithelial cells and remain to initiate the enteroepithelial cycle, whereas others penetrate through the mucosa to begin development in the lamina propria, mesenteric lymph nodes, other distant organs and white blood cells (extraintestinal cycle). In hosts other than felines, only the extraintestinal cycle exists; and sporozoite entry into host cells results only in asexual endodyogeny with the production of tachyzoites (15). After entry into a host cell, there is a variable and parasite dependant lag period before the parasite begins to divide (15). The tachyzoites proliferate within vacuoles of the host cell and continue to proliferate until 8-16 organisms accumulate after which cell lysis occurs (15, 18). The tachyzoite is often crescent shaped with a pointed anterior end and a rounded posterior end (approximately 3µm X 7µm). Ultrastructural studies have shown the tachyzoite to be rather complex consisting of

various organelles (e.g.: nucleus, ribosomes, golgi apparatus, mitochondria, etc.) and inclusion bodies (17). The efficiency of invasion and growth vary with both the strain of *T. gondii* and the type of host cells that are infected. Tachyzoites are present in the acute stage of infection and during recrudescence of a chronic infection. Tachyzoites may accumulate in host cells for a prolonged period of time without forming true cysts. As immunity develops in the host, tachyzoite multiplication slows down and bradyzoites are formed in tissue cysts, which are infectious to all mammals.

1.2.4 – Tissue Cyst Formation

Tissue cysts vary considerably in size (10-100µm) and contain up to 3000 bradyzoites (15, 17). Bradyzoites multiply slowly by endodyogeny primarily in the skeletal muscle, the myocardium and the brain and co-exist within these host cells for long periods of time. They can also be found to a lesser extent in visceral organs such as the lungs, liver and kidneys. Intact tissue cysts do not cause any serious harm to the host and can persist for the life of the host without causing an inflammatory response (15). The cyst is characteristic of chronic infection but can be isolated as early as 6-12 days after infection. In immunodepressed mammals, tissue cysts can rupture resulting in unrestricted tachyzoite proliferation that can overwhelm and kill the host.

1.3 – EPIDEMIOLOGY

Toxoplasma infection is a worldwide zoonosis occurring wherever felines are present (1, 19, 20). It is estimated that up to 1% of cats worldwide are infected (1). The organism has the capacity to infect all orders of mammals including herbivores,

omnivores and carnivores. The presence of tissue cysts in meat for human consumption is high (e.g.: lamb, beef) (1, 19, 20). Since *T. gondii* organisms are rarely isolated or directly demonstrated in humans suffering from toxoplasmosis, serological tests are usually needed to confirm the presence of infection. In many populations such as French Canada, El Salvador, Tahiti and France, seropositivity can exceed 90% by the fourth decade of life (20). High prevalence of infection in France and French Canada (85%) have been linked to a preference for eating raw or undercooked meat, while high prevalence in Central America has been linked to the presence of large numbers of stray cats in a climate favoring the survival of oocysts (1). The incidence of infection is not significantly different between sexes (19, 20). Infection is more common in warm climates and at lower altitudes than in cold climates and mountainous regions (19, 20). This is likely due to the number of feral cats and the relative intolerance of oocysts to extreme environmental conditions. The overall seroprevalence in the United States determined by the third National Health and Nutritional Assessment Survey (NHANES III) between 1988 and 1994 was found to be 22%. The seroprevalence among women of childbearing age (15 to 45 years) was 10% to 15%.

1.4 – CLINICAL PRESENTATION

How *Toxoplasma* presents likely involves a critical balance between the development of a protective immune response (21-25) and the down regulation or suppression of the immune response (26-40). This balance is clearly influenced by age, pregnancy and immunosuppression. Toxoplasmosis has five primary clinical presentations: (A)

acute acquired illness in the immunocompetent patient, (B) chronic illness, (C) acute or reactivated illness in the immunocompromised patient, (D) congenital infection and (E) ocular infection (19).

1.4.1 – Acute Infection in the Immunocompetent Patient

Acute *Toxoplasma* infection is acquired by eating tissue cysts in meat or ingesting oocysts from soil contaminated with feline feces. Organisms entering the intestinal wall are disseminated via lymphatics and hematogenously and multiply in a variety of tissues (40). The degree of tissue damage that occurs depends on the duration and intensity of this unrestricted multiplication stage. The development of immunity eventually stops the multiplication of tachyzoites (40). A relatively brief period of multiplication results in subclinical infection or mild illness. Prolonged multiplication from large inocula and/or with the slow development of immunity, can lead to moderate or severe illness and death (19).

In the majority of humans, acute infection is an asymptomatic and mis-diagnosed infection. Only 10-20% of cases of *Toxoplasma* infections in adults are symptomatic (20). When symptoms develop, toxoplasmosis most often presents itself as a mild to moderate cervical lymphadenopathy, but any or all lymph node groups may be enlarged. The clinical picture may resemble infectious mononucleosis or cytomegalovirus infection, but acute toxoplasmosis is probably responsible for no more than 1% of mononucleosis syndromes (19). Fever, malaise, myalgia, fatigue, headache, sore throat, maculopapular rash sparing palms and soles,

hepatosplenomegaly, atypical lymphocytes and hilar mediastinal lymphadenopathy may be present (19).

If poorly controlled, acute acquired toxoplasmosis may be associated with central nervous system (CNS) disease, with or without concomitant involvement of the lung, heart, liver and muscle (40). When this occurs, patients present with non-specific neurological symptoms such as confusion, seizures, disorientation and lethargy. Patients may also complain of symptoms including fever, arthralgia, myalgia and maculopapular rash (40). Cardiac involvement may be associated with congestive heart failure and electrocardiographic abnormalities due to arrhythmias (40). *Toxoplasma* has also been implicated as a cause of diffuse interstitial pneumonitis in previously healthy adults. Other complications include acute acquired toxoplasmic retinochoroiditis and localized brain infection (40).

Cases of acute *T. gondii* infection from common-source outbreaks (e.g.: water-borne outbreak in Victoria B.C., Canada, 1995) (41) and experimental infections in animals provide insight into both the pathogenesis of this disease and the development of immunity. The major pathogenic factor is the proliferation of tachyzoites that destroy host cells more quickly than they can be repaired and regenerated. Based upon observations in animals (42), it is likely that inoculum size and *Toxoplasma* virulence factors influence pathogenicity in humans (42). The appearance of variable microbial pathogenicity might also be explained on the basis of variations in the host immune response.

1.4.2 – Chronic Infection

In immunocompetent individuals, *T. gondii* is ordinarily kept at bay by cell-mediated-immunity (31). However, once introduced, this organism is rarely if ever eradicated. Chronic *Toxoplasma* infection results when immunity builds up sufficiently to depress tachyzoite proliferation. This coincides with the formation of tissue cysts that remain intact for years and produce no obvious clinical effect. Evasion of host immunity and the ability to live inside cells without destroying them, for periods of months to years, are the biological hallmarks of bradyzoites within cysts. The number of bradyzoites within large cysts may be as high as a thousand. When syndromes do occur in chronic toxoplasmosis they are usually due to very high cyst densities within host tissues. Occasionally, a cyst wall breaks down releasing bradyzoites. Most released bradyzoites are destroyed by the host's immune response, however some of them evade immune-mediated killing and form new cysts. Bradyzoite rupture or destruction elicits an intense hypersensitivity reaction that in the brain, for example, can gradually be replaced by glial cell nodules. If many such nodules are formed, the host may develop symptoms of chronic encephalitis, with spastic paralysis in some cases (40). Sporadically, lesions may also develop in the eye, related circumstantially to tissue cyst rupture. The release of antigenic materials can lead to local intense inflammation in the retina causing fibrosis and blind spots. Extensive infection of the central macular area, can lead to complete blindness (32-36). Myocarditis, associated with permanent heart damage, can also occur as a result of chronic toxoplasmosis.

In most cases, chronic toxoplasmosis lesions are self-limited due a protective immune response. The persistence of immunity is demonstrated by the fact that the majority of bradyzoites released from a ruptured tissue cyst are killed extracellularly and that few or no new satellite lesions arise (37, 38). Immunosuppressive agents (e.g.: high-dose corticosteroids) and progressive immunocompromising illness (e.g.: HIV) can permit tachyzoite proliferation to resume (39).

1.4.3 – Acute or Reactivated Illness in the Immunocompromised Patient

Acute or reactivated disease can cause fatal illness in immunocompromised hosts such as those infected with HIV and post-transplant patients (1-3, 19). Recrudescence of active toxoplasmosis occurs in approximately 25% of *Toxoplasma*-infected AIDS patients and in patients receiving immunosuppressive therapies (39).

A hallmark of reactivated toxoplasmosis is CNS involvement including retinochoroditis and toxoplasmic encephalitis. The manifestations of toxoplasmic encephalitis vary widely from a subtle and subacute process that evolves over weeks-to-months to an acute confusional state with or without focal neurological deficits accompanied by headache. The clinical manifestations of toxoplasmic encephalitis are generally classified as one of two patterns: focal versus diffuse (43). In the former group, focal abnormalities such as focal seizures, hemiparesis, hemiplegia, hemisensory loss, cerebellar tremor, homonymous hemianopsia, cranial nerve palsies, diplopia, blindness, personality changes and severe headaches are found (43). More diffuse signs of CNS dysfunction in the second group include weakness, myoclonus,

confusion, lethargy, disorientation, headache and coma. As diffuse disease progresses, some patients can develop focal signs (44). Focal neurological defects may arise as a result of outright tissue destruction (e.g.: necrotizing lesions) or as a result of accompanying edema or vasculitis (44).

Toxoplasma can infect any cell in the brain but specific focal neurological symptoms vary depending on the areas of the brain affected. However, there is a tendency for *Toxoplasma* to cause disease in the brain stem, basal ganglia and the pituitary gland (45). Brain stem involvement causes a spectrum of neurological symptoms that includes ataxia, limb dysmetria and cranial nerve palsies (46). Choreiform movements and choreoathetosis have been found in patients with basal ganglia infection. Acquired hydrocephalus and blindness may also develop.

1.4.4 – Congenital Infection

Congenital toxoplasmosis is the result of maternal infection (usually asymptomatic) acquired, during or just prior to pregnancy (1-3, 19, 47, 48). More specifically, congenital disease occurs when organisms infect the placenta, and after a lag period, the unborn fetus. The incidence and severity of infection caused by *T. gondii* is directly related to the trimester during which the organism is acquired. Primary infection in the first three months of pregnancy carries a 15% risk that the fetus will be infected. The probability increases to 59% for primary infection during the third trimester (49). However, the severity of congenital disease is the highest in the first trimester and decreases progressively with gestational age. The risk of fetal infection

is highest in the third trimester due to the fact that the placenta has attained its maximum size, facilitating passage of parasites to the fetus, while the risk of congenital disease is highest in the first trimester when the fetus' vital organs are developing (47, 48). Detection of maternal infection early in pregnancy allows prospective mothers to consider abortion of the fetus to avoid the substantial risk of spontaneous abortion, stillbirth and mental retardation of the child (19, 47-49). In order to assess accurately the risk of transmission of *T. gondii* to the fetus, it is imperative to determine when the pregnant woman acquired the infection with respect to the date of conception. If acquisition of *T. gondii* predates conception by more than three to four weeks, the incidence of congenital toxoplasmosis is extremely low (19, 47-49). The recognition of congenital infection in children is usually quite difficult because the vast majority of infections in immunocompetent hosts (including pregnant women) are asymptomatic. Furthermore, the sequelae of congenital infection may only become apparent many years after birth. Most of the signs and symptoms of congenital toxoplasmosis are non-specific and may mimic the syndromes caused by congenital infection with herpes simplex virus (HSV), cytomegalovirus (CMV) and rubella virus (19, 47-49).

1.4.5 – Ocular Infection

Ocular *Toxoplasma* infection is an important cause of chorioretinitis that usually results from congenital infection. Patients are often asymptomatic until the second or third decade of life, when lesions develop in the eye due to cyst rupture and subsequent release of tachyzoites and bradyzoites. Chorioretinitis is characteristically

bilateral in patients with congenital infection but is usually unilateral in people who acquired *Toxoplasma* infection after birth (32, 33).

1.4.6 – Standard Therapy

The standard treatment for toxoplasmosis involves the use of a sulphonamide (e.g.: sulphadiazine, sulphamethoxazole) and a diaminopyrimidine (e.g.: pyrimethamine, trimethoprim). Each drug has a significant individual inhibitory effect and they act synergistically to deter parasite growth and propagation. Sulphonamides act against the incorporation of *p*-aminobenzoic acid into pteroylglutamic acid (folic acid) and the diaminopyrimidines work against dihydrofolic reductase, reducing the transformation of dihydrofolic into tetrahydrofolic (folinic) acid (39). This chemotherapy can be highly effective even in congenitally infected babies. These standard chemotherapeutic regimes, whether sulphadiazine with pyrimethamine or sulphamethoxazole with trimethoprim, radically reduce the number of organisms associated with lesions, so that after 3 weeks of treatment, only tissue cysts remain. After several months of treatment, lesions may be undetectable by histological methods (39).

1.5 – TOXOPLASMA DIAGNOSTIC TECHNIQUES

The diagnosis of toxoplasmosis is currently based primarily on serology, but techniques for the detection of parasite nucleic acids or antigens are gaining in popularity (50-52).

1.5.1 – Parasite Isolation

Only rarely can the diagnosis of toxoplasmosis be confirmed by the isolation of parasites from patient specimens or by histological identification. Isolation from biopsy specimens is occasionally possible by intraperitoneal inoculation into infection-free mice. However, definitive results may not be available for 6-8 weeks. Inoculation of cell lines (e.g.: human embryonic lung cell line) that are subsequently examined by immunofluorescence can produce more rapid results, but this technique is less sensitive (53, 54). Although these methods cannot reliably differentiate between active and chronic infection, they have been used in the investigation of reactivated toxoplasmosis in AIDS patients (55) and in fetuses at risk of acquiring congenital toxoplasmosis (56). Interpretation of culture results from *Toxoplasma* tachyzoites isolated from AIDS patients may be particularly problematic when prophylactic antimicrobial agents with activity against *T. gondii* are used (e.g.: Septra[®]) (57).

1.5.2 – Serological Techniques

1.5.2.1 – The Sabin and Feldman dye test

The test was developed in 1948 by Sabin and Feldman and is the accepted reference assay for the detection of *Toxoplasma* specific antibodies (11). In this assay, living tachyzoites are incubated with serum containing *Toxoplasma* antibody and complement. If *T. gondii* specific antibodies are present, the organism will lyse and fail to stain with alkaline methylene blue. The titre is defined as the serum dilution at which half of the organisms are lysed. This titre can be converted into international units (IU) by comparison to a reference serum. Unlike other assays, the dye test can

simultaneously detect both complement-fixing IgG and IgM. Patients become dye test positive within 1-2 weeks of exposure to the parasite and titres reach a peak by 8 weeks. Low titres commonly persist for life. Although it is difficult to perform, the Sabin and Feldman dye test is both highly sensitive and specific. There are however certain limitations to this assay. First, reference laboratories have reported cases of false-negatives with the use of this test (58). Second, the titres of the dye test do not correlate with the clinical stage of disease and thus cannot differentiate between acute and chronic infection. Finally, since the test requires live organisms, it is considered a safety hazard. This laboratory consideration has contributed to the replacement of the dye test with less hazardous tests (19).

1.5.2.2 – The Indirect Hemagglutination Test

In 1957, Jacobs and Lunde described an indirect hemagglutination test (IHAT) for toxoplasmosis (59). In this test, patient serum is incubated with red blood cells (RBCs) previously sensitized with a soluble *Toxoplasma* antigen preparation. If the patient has antibodies to *Toxoplasma*, the RBCs will agglutinate. The IHAT is simple to perform, inexpensive, applicable for both humans and animals and practical for testing large numbers of serum samples. Technical disadvantages of the assay include variability in RBC and antigen quality (19). Furthermore, the IHAT detects antibodies later than the Sabin and Feldman dye test and frequently does not detect infection in pregnant women and newborn children (19, 60, 61). The IHAT has mainly been used for survey purposes to determine either the incidence or prevalence of infection in populations (62). When directly compared with other assays in veterinary studies, the

IHAT does not perform as well as the ELISA, the direct agglutination test (DAT) or the latex agglutination tests (LAT) (63, 64).

1.5.2.3 – The Direct Agglutination Test

Although the DAT was first described in 1959 by Fulton and Turk (65), the test lacked sensitivity and specificity until modifications developed by Desmonts and Remington were incorporated in 1980 (66). This test uses formalin-fixed intact tachyzoites and agglutination of parasites occurs if *Toxoplasma* antibodies are present in the test sample. The test should only be used for detection of IgG antibodies. This assay has proved to be sensitive and inexpensive as a screening test (67). Although, naturally occurring IgM antibodies can induce non-specific agglutination, this problem can be overcome by the use of 2-mercaptoethanol in the serum diluent (66). However, in direct comparison with other tests such as the IHAT, the ELISA, the LAT and the immunofluorescent antibody test (IFAT) (67), the DAT does not perform well enough to be used as a screening test on its own. False positive results are a particular problem in congenital infection (68), toxoplasmic lymphadenopathy (69) and toxoplasmic encephalitis (70).

1.5.2.4 – The Latex Agglutination Test

In the LAT, antigens derived from disrupted tachyzoites are fixed to latex beads. When beads and patient serum containing specific IgG antibody are mixed, visible agglutination occurs (71). This test is easy to perform, inexpensive and can be used in both animals and humans. However, low sensitivity and specificity have been

observed when testing immunocompromised patients (72, 73). Also, false positive reactions can occur when compared to the Sabin and Feldman dye test (72, 73). These false positives have been attributed to non-specific IgM reactions (74-76). Although at least one commercial kit is available (Toxolates Fumouze), latex agglutination testing in pregnancy, AIDS patients and immunosuppressed patients is plagued by both false negative (77, 78) and false positive results (79, 80). Although the LAT can detect *T. gondii* infection much earlier in the infection than the ELISA, the ELISA technique is more useful in detecting prior infections (79, 80).

1.5.2.5 – The Indirect Fluorescent Antibody Test

The IFAT for toxoplasmosis uses whole formalin-fixed tachyzoites that are air-dried on microscope slides as antigen (81). These slides are incubated with serial dilutions of the patient's serum and a specific antigen-antibody interaction is detected by a fluorescein-conjugated secondary antibody against serum IgG or IgM isotypes or total immunoglobulin. Since its introduction, the IFAT has been widely used by public health laboratories in both Canada and the United States as an alternative to the Sabin and Feldman dye test, eliminating the need for live parasites (19). Though the IFAT is more widely available, the dye test is quantitatively more reliable and the ELISA is more sensitive (79, 82). Antibodies in the IgM-IFAT appear as early as 5 days after infection, rise rapidly and then fall to low titres, usually disappearing within a few weeks or months. However, low titres may persist for over one year in some patients. The IFAT is negative in some immunocompromised patients, most patients with ocular toxoplasmosis and approximately 75% of infants with congenitally acquired

infection (81). In some patients with acute acquired toxoplasmosis, high levels of IgG anti-*Toxoplasma* antibodies interfere with the IgM-IFAT and cause false-negative results (83). False-positives may occur in patients with antinuclear antibodies (83, 84). Direct comparison studies suggest that the IFAT is a good predictor of infection but cannot replace ELISA testing (67, 79).

1.5.2.6 – Enzyme Immunoassays

Enzyme immunoassays (EIAs) or enzyme-linked immunosorbent assays (ELISAs) have become the most widely used tests for toxoplasmosis screening by public health laboratories. Several commercial kits are available for the detection of IgM (IgM kits: the Palo Alto Medical Foundation double sandwich IgM ELISA, the Centers for Disease Control and Prevention EIA IgM, the Abbott IMx Toxo IgM version 1, the Abbott IMx Toxo IgM version 2, the Abbott Toxo-M EIA, the BioMerieux Vitek VIDAS Toxo IgM, the BioWhittaker Toxocap-M, the Gull Toxo IgM and the Sanofi Diagnostics Pasteur Platelia Toxo IgM) and IgG (IgG kits: Cordia-T-IgG, Toxo Bio-EnzaBead-IgG, Tozoelisa-IgG, Platelia Toxo IgG) (4, 85). In these assays, soluble *T. gondii* antigen is absorbed onto a matrix. Patient serum is then incubated with the antigen, followed by an enzyme-labelled antihuman conjugate and then substrate. The resulting colour change is read spectrophotometrically, allowing ELISA tests to be both qualitative and semi-quantitative. There are many variations on this simple method. The sensitivity of conventional ELISA methods can be improved by the use of the double-sandwich format.

Although the IgM tests generally have a high degree of accuracy, false-positive results certainly occur (4, 29). These false-positive results are most probably due to the presence of rheumatoid factor in serum (83, 86). False-negative results are also common due to competitive inhibition by *Toxoplasma*-specific IgG (87). Preabsorption of serum with a *Staphylococcus/Streptococcus* preparation removes IgG and IgA and eliminates many, but not all, of these false negative reactions (87). Recently, several ELISA tests for avidity of *Toxoplasma* IgG antibodies have been described to facilitate discrimination between recently acquired and distant infections (88-90). To date the only commercial avidity test is available in Europe (88, 89). Results are based on the measurement of functional affinity of specific IgG antibodies. IgG affinity is initially low after primary antigenic challenge, but increases during subsequent weeks and months by antigen-driven B cell selection.

1.5.2.7 – Western Blot

The western blot (WB) is a variation of EIAs. Antigen is electrophoresed through an acrylamide gel and the proteins are separated according to their size. It is electrophoresed again to allow the transfer of the proteins to a nitrocellulose matrix that is processed to visualize the reaction. The WB can be used to detect antibodies directed against individual parasite antigens/proteins but has failed as a routine diagnostic test (91-95). WB has also been used to demonstrate different antibody profiles at different stages of *T. gondii* infection in congenital infection. Two major studies recently conducted in France concluded that WB could be used as a complimentary test but could not replace the more sensitive serologic assays

(described above) (96, 97). Unfortunately, interpretation of WB is complicated by naturally occurring antibodies in non-immune sera that are reactive with *Toxoplasma* antigens (98). The technique is not quantitative and accurate measurement of antigen molecular weights can be a problem (99-103).

1.5.3 – PCR

Numerous PCR assays have been developed that target predominantly four genes. These assays have typically been tested with small numbers of human specimens obtained from various body sites including cerebrospinal fluid, bronchoalveolar lavage fluid, brain, liver, amniotic fluid, ocular sections, lymph nodes, heart and muscle tissues (104-126). PCR-based assays are theoretically very sensitive and specific.

The first application of PCR to the detection of *T. gondii* followed the identification of B1, a gene with more than 35 copies in the *T. gondii* genome (127). Several groups have subsequently targeted this gene using primers located at different sites (106, 109, 112, 115) or primer pairs (113). The gene encoding the major surface antigen, P30, has been the target of two independent PCR-based assays (128, 129), one of which used nested PCR to increase assay sensitivity (129). The small-subunit rRNA gene sequence (~110 copies/parasite) has been used for several other PCR assays (109, 121, 130, 131). Another repetitive DNA sequence, TGR1_E, has also been targeted (132). To differentiate *T. gondii* from closely related coccidians with similar host ranges, riboprinting (restriction enzyme analysis of PCR-amplified small subunit

rRNA gene sequences) has also been used (133). Compared with the histologic detection of cysts in cardiac tissue, PCR is more sensitive, although this test cannot distinguish between quiescent cysts and free tachyzoites resulting from active infection (126). Although high sensitivity and specificity can be achieved with PCR, these assays are limited by tissue distribution of parasites in some clinical situations. PCR-based assays clearly work best with tissue samples. Sensitivity can drop significantly when readily accessible tissues (e.g.: blood, CSF) are used, as is the case for toxoplasmosis.

1.6 – ANTIGEN DETECTION AS A DIAGNOSTIC APPROACH

1.6.1 – Introduction

Historically, the diagnosis of most human parasitic diseases has been based on the direct demonstration of the organisms in tissues, body fluids or fecal specimens (134, 135). Such investigations are limited in many diseases by the difficult accessibility of the parasite and the need for highly skilled microscopists or pathologists. Direct demonstration techniques can be highly specific but almost always have very low sensitivity because the parasites can be of a low number. As a result, both antibody and antigen detection tests have been investigated as alternatives. These serologic methods are particularly appropriate if invasive procedures would otherwise be needed to obtain specimens for examination such as in toxoplasmosis, cysticercosis, echinococcosis, toxocariasis and trichinosis (134, 135, 136). The limits of many of the currently used antibody-based serologic assays have already been described.

1.6.2 – Antigen Detection Assays

Antigen detection assays offer an alternative solution to many of the problems associated with antibody-based diagnostics. The detection of parasite antigens should be a direct indication of active infection and should be useful to demonstrate response to therapy. These assays can be highly specific and can use either polyclonal or monoclonal antibody cocktails in a simple immunoassay format that can be quickly performed by relatively unskilled laboratory personnel.

In theory, a wide range of parasite antigens might be of use in antigen detection assays including membrane surface molecules, molecules associated with internal structures/organelles or secretory/excretory products. The “ideal” antigen for diagnostic purposes would be one that is stage specific, present in large amounts in body fluids and non-immunogenic. This last feature would be desirable to prevent rapid immune clearance and to simplify detection (i.e.: avoid the complexities of dealing with circulating immune complexes). Unfortunately, all antigen detection assays are currently based upon either monoclonal or polyclonal antibodies, which have to be generated *in vivo*. As a result, the antigens targeted by these assays are immunogenic by definition.

There is a large body of literature that deals with the development of antigen detection assays for parasitic infections (reviewed in table 1). Much work has been accomplished during the course of the last 10 years resulting in the release of several commercial kits (136, 137) that now replace or complement morphologic and

serodiagnostic tests for organisms such as *Cryptosporidium*, *Entamoeba*, *Filaria*, *Giardia*, *Plasmodium*, *Schistosoma* and *Trypanosoma* species.

Table 1: Development of Antigen Detection Assays for Parasitic Infections

DISEASE	ETIOLOGIC AGENT	RESEARCH & DEVELOPMENT	PROBLEM WITH SERODIAGNOSIS	REFERENCES
Amebiasis	<i>E. histolytica</i>	<i>E. histolytica</i> II antigen in serum and stool (EIA)	1) Titre may not indicate acute/current infection	139-143
Cryptosporidiosis	<i>C. parvum</i>	Coproantigen EIA, IIF, WB reverse passive hemagglutination, PCR	1) Unable to distinguish between acute & chronic infections	144-149
Cysticercosis	<i>T. solium</i>	Ag-detection in CSF, sera (EIA)	1) Ab-EIA for neurocysticercosis - low predictive value 2) cross-reactions with Filaria infections & Hydatid Disease 3) Cannot distinguish between acute & chronic infections	150-155
Echinococcosis	<i>E. granulosus</i> <i>E. multilocularis</i>	Coproantigen mAb-EIA, PCR	1) Sensitivity ranges from 60-90% 2) High amount of false positives 3) Some patients do not produce Ab 4) Immune complexes	156-163
Fasciolosis	<i>F. hepatica</i>	mAb-coproantigen & serum based EIA	1) Ab cross-react with Schistosoma antigens	164-172
Filariasis	<i>W. bancrofti</i> <i>B. malayi</i>	Serum based EIA	1) Immune complexes	173-187
Giardiasis	<i>G. lamblia</i> <i>G. duodenalis</i>	Coproantigen EIA, IIF (soluble antigen)	1) Low sensitivity	188-201
Leishmaniasis	<i>L. donovani</i> <i>L. major</i> <i>L. tropica</i> <i>L. infantum</i> <i>L. brasiliensis</i>	<i>L. donovani</i> mAb EIA (~88% sensitivity) PCR WB	1) Not useful for cutaneous Leishmaniasis 2) Cannot determine the infecting species 3) cross-reactivity with Chagas' Disease	202-214
Malaria	<i>P. falciparum</i> <i>P. malariae</i> <i>P. ovale</i> <i>P. vivax</i>	Ag-detection test HRP-II (EIA) Dipstick Ag-detection test HRP-II (ParaSight-F) Dipstick Ag-detection test HRP-II (ICT Malaria Pf) Tests for lactate dehydrogenase (OptiMAL) Tests for various transcription factors (PCR)	1) Cannot distinguish between acute & chronic infections	215-235
Onchocerciasis	<i>O. volvulus</i>	Ag-detection EIA using serum, skin, urine PCR	1) IgG & IgE are detected in sera - not specific for presence or extent of infection 2) cross-reactions with Filaria infections	236-244
Schistosomiasis	<i>S. manoni</i> <i>S. japonicum</i> <i>S. haematobium</i>	Ag-detection EIA using stool & urine Identifies circulating cathodic/anodic antigens (i.e.:CCA & CAA)	1) Presence of Ab only indicate infection at some time & cannot be correlated with worm burden, egg production clinical status or prognosis 2) Immune complexes	245-261
Toxoplasmosis	<i>T. gondii</i>	Ag-detection EIA in sera, CSF PCR Western Blot	1) Cannot distinguish between active & latent infection 2) 3% of AIDS patients don't produce Ab 3) IgM lingers for up to 2 years post-infection	51, 52, 91-95 104-126 262-275
Trichinellosis	<i>T. spiralis</i>	Ag-detection EIA PCR Western Blot	1) Humans rarely develop Abs until 3-5 post infection 2) Serologic tests remain positive for months - years	276-284
Trypanosomiasis	<i>T. cruzi</i> <i>T. brucei</i> <i>T. gambiense</i> <i>T. rhodisiense</i> <i>T. vivax</i>	Indirect agglutination card test (not for <i>T. cruzi</i>) mAb-EIA in urine, blood, serum PCR	1) Cannot distinguish between acute & chronic infections	285-299

LEGEND

EIA: Enzyme Immunoassay
IIF: Indirect Immunofluorescence

WB: Western Blot
PCR: Polymerase Chain Reaction

1.6.3 – Toxoplasma Antigen Detection Assays

Soluble *T. gondii* antigens have been detected in the urine and serum of acutely infected mice (263-266). The successful detection of antigen in human serum was first recorded in 1977 (262). Since that time, many groups have attempted to exploit the potential of this approach with highly variable success. Although *T. gondii* specific antigens can be found mainly in serum (52, 91-100, 106, 117, 119, 262, 267-270) and CSF (108-111) in humans, the overall sensitivity of these assays has ranged from 4-75% (versus reference tests such as the Sabin-Feldman dye test, 90-100%). To date, more than 20 antigens have been described ranging in size from 4-115 kDa (91-95). Several have been proposed as markers for acute (97 kDa) or chronic infection (22 kDa, 30 kDa, 35 kDa, 43 kDa) (92). However, the ability to detect these antigens with high sensitivity is low for diagnostic purposes. Furthermore, the large majority of the assays derived so far are based upon polyclonal sera. Our study uses monoclonal antibodies, which will allow us to dramatically increase assay sensitivity while still achieving a high degree of specificity. Ultimately, an assay capable of discriminating between acute and chronic infection will be of great diagnostic value.

CHAPTER 2

MATERIALS & METHODS

2.1 – ANTIBODIES

2.1.1 – Monoclonal Antibodies

A panel of five novel mAbs against *Toxoplasma* tachyzoite antigens were provided by Dr. Gary E. Ward, University of Vermont (Burlington, Vermont, USA) (Table 2).

Table 2: Anti-*Toxoplasma* Mouse Monoclonal Antibodies

CLONE	ISOTYPE	ANTIGEN	TACHYZOITE LOCATION
45.15	IgG2a (κ)	70 kDa	Inner Membrane Complex
A3.2	IgG1, 2a, 3 (κ)	34 kDa	Dense Granules
17.9	IgG2b, IgG1 (κ)	31 kDa	Dense Granules (GRA6)
C8.4	IgG1 (κ)	57 kDa	Surface/External Antigens
B3-90	IgG2a (κ)	60 kDa	Internal Antigen of Apical Complex

2.1.2 – Specimens

Serum samples used in these studies were obtained from the National Centre of Parasite Serology (NCPS – Dr. Bouchra Serhir – Montreal, Quebec, Canada), the Ontario Public Health Lab (OPHL – Dr. Neal DenHollander – Toronto, Ontario, Canada) and the Laboratoire de Santé Publique du Québec (LSPQ – Dr. Michel Couillard – Montreal, Quebec, Canada). Samples from children in St. John's, Newfoundland that were *T. gondii* IgG negative were used as negative controls (n=103). The great majority of the samples had been stored at –20°C for less than 2 years. The remainder of the samples had been at –20°C for a period of 3-10 years. The serological tests performed by the sample providers were: the IgM immunofluorescence antibody test (IgM-IFAT: Toxo-IgM IFAT, Gen Bio, San

Diego, California, USA) and the direct IgG ELISA (IgG-ELISA: Platelia-TOXO-IgG Solid Phase EIA, Bio-Rad, Mississauga, Ontario, Canada). The results of the IgM-IFAT and IgG-ELISA tests were used to group sera into four serologic “profiles” for purpose of comparison: G^+/M^+ (n=211), G^+/M^- (n=166), G^-/M^+ (n=35), G^-/M^- (n=110). IgM titres ranged from 1:4 to 1:2048 and IgG titres ranged from 1:256 to 1:16384. Only limited information regarding gender, age or reason for original *Toxoplasma* testing was available for most of these subjects.

Additional samples used to perform an initial assessment of assay specificity (n=50) were obtained from the NCPS “Gold Standard” reference serum bank. These samples included sera from subjects with: amebiasis (n=5), cysticercosis (n=6), filariasis (n=10), hydatid disease (n=10), malaria (n=6), schistosomiasis (n=9) and trichinosis (n=4).

2.2 – ANTIGENS

2.2.1 – Antigen Preparation

Toxoplasma tachyzoites (RH lab strain) were kindly provided by Dr. Gary E. Ward (University of Vermont) and grown in human foreskin fibroblast (HFF) cell monolayers (ATCC, Manassas, Virginia, USA). Following cell lysis, the supernatant was discarded and parasites were pelleted by centrifugation (10,000xg for 10 minutes). The tachyzoite pellet was resuspended in sterile phosphate buffered saline (PBS; 3mM KH_2PO_4 , 12 mM Na_2PO_4 , 0.116M NaCl, pH 7.2) and sonicated on ice (Kontes High Intensity Ultrasonic Processor 40-Watt model) (Vineland, New Jersey,

USA) to produce a crude whole tachyzoite antigen preparation. Briefly, 5 rounds of ten 3-second bursts with an output of 100mHz were needed to fully solubilize the pellet. Antigen preparation protein concentration was quantified by a modified Bradford Assay (Bio-Rad, Mississauga, Ontario, Canada).

2.3 – DIRECT ANTI-*TOXOPLASMA* IgG ELISA

We first investigated whether or not sera known to contain anti-*Toxoplasma* antibodies would recognize the *Toxoplasma* antigen preparation. Round-bottomed, 96-well polystyrene microtitre plates (Greiner, Longwood, Florida, USA) were coated with 100µl per well of the antigen preparation at concentrations of 0µg/ml, 0.5 µg/ml, 1.0 µg/ml and 2.0 µg/ml in 0.1M carbonate/bicarbonate buffer (pH 9.6) overnight at 4°C. Following overnight incubation, the microtitre plates were washed 4 times in PBS/0.05% Tween-20 (PBS-T, pH 7.2) and blocked with 100µl per well of 2% bovine serum albumin (BSA) in PBS-T (BSA, Cohn fraction V powder in PBS; Sigma, Oakville, Ontario, Canada) and incubated at 37°C for 1 hour. Microtitre plates were then washed 4 times and 100µl of diluted *Toxoplasma* sera in 2% BSA/PBS-T (1:100, 1:200, 1:400) was applied to the appropriate wells and the plates were incubated at 37°C for 1 hour. After 4 washes, 100µl of peroxidase conjugated affinity-purified goat anti-human IgG (1:5000 in blocking solution, Kirkegaard & Perry Labs Inc., Gaithersburg, Maryland, USA) was added to each well and the plates were incubated at 37°C for 1 hour. Microtitre plates were washed an additional 4 times and freshly prepared ABTS solution (ABTS solution: 5ml 10X ABTS concentrate, 45ml ddH₂O, ABTS 50mg pellet, 2µl of 30% hydrogen peroxide per 5ml

of ABTS solution; Sigma) was added to each well. The ABTS kit was used according to the manufacturer's instructions. The optical density values were measured at a wavelength of 405nm (OD₄₀₅) with an automatic ELISA plate reader (Titertek Multiskan MCC/340) (Titertek, Huntsville, Alabama, USA) after 15 minutes of incubation at room temperature.

2.4 – ANTIGENEMIA ASSAYS

2.4.1 – First Generation *T. gondii* Antigen Detection ELISA

96-well microtitre plates (Greiner) were coated with either 50 µl of a pool of the 5 mAbs (2 µg/ml of each mAb) (Table 2) or 50 µl of an individual mAb at a concentration of 10 µg/ml in 0.1M carbonate/bicarbonate buffer (pH 9.6) overnight at 4°C. Following overnight incubation, the microtitre plates were washed 4 times in PBS/0.05% Tween-20 (PBS-T, pH 7.2) and blocked with 100µl per well of 2% BSA blocking solution in PBS-T and incubated at 37°C for 1.5 hours. Microtitre plates were then washed 4 times and 50µl of TCA treated serum (see below) was applied to the appropriate wells and the plates were incubated at 37°C for 2 hours. After 4 washes, 50µl of purified IgG (see below) at a concentration of 20µg/ml in 2% BSA was added to the wells and incubated at 37°C for 2 hours. Following another 4 washes, 50µl of peroxidase conjugated AffiniPure donkey anti-goat IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pennsylvania, USA) was added to each well at a final dilution of 1:5000 in 2% BSA and incubated at 37°C for 2 hours. After 4 washes, 50µl of ABTS solution (prepared as previously described) was added to each well and microtitre plates were incubated at room temperature.

OD₄₀₅ values were measured with an automatic ELISA plate reader (Titertek) after 30 minutes and 60 minutes of incubation at room temperature.

2.4.2 – Second Generation *T. gondii* Antigen Detection ELISA

The first five steps of the first generation ELISA were used to design the second generation ELISA. Following the fifth step, microtitre plates were washed 4 times and 50µl of biotinylated AffiniPure F(ab')₂ fragment donkey anti-goat antibody (Jackson ImmunoResearch Laboratories Inc.) was added to each well at a final dilution of 1:20000 in 2% BSA and incubated at 37°C for 2 hours. After 4 washes, 50µl of streptavidin-horseradish peroxidase conjugate (Gibco BRL, Burlington, Ontario, Canada) was added to each well at a final dilution of 1:1000 in 2% BSA and incubated at 37°C for 2 hours. Spectrophotometric development was performed as described in the first generation assay.

2.4.3 – TCA Treatment of Serum Samples

A solution of 30% (w/v) trichloroacetic acid (TCA) (30g of TCA (ICN Laboratories, Costa Mesa, California, USA) in 100ml of ddH₂O) was prepared. For example, 40µl of 30% TCA was added to 60 µl of sera (12% TCA final concentration). TCA treated sera was incubated at room temperature for 20 minutes then centrifuged for 3 minutes at 10,000xg. The supernatant was then collected and applied to the wells of the microtitre plates.

2.4.4 – Antibody Purification on Protein A Columns

A Protein A column (Pharmacia, Peapack, New Jersey, USA) was used to purify IgG from goat anti-*Toxoplasma gondii* polyclonal antiserum (VMRD, Inc., Pullman, Washington, USA). The column was first washed with 2M urea (Gibco BRL), 1M LiCl (Gibco BRL) and 100mM glycine (pH 2.5) (Gibco BRL) and then antiserum was applied. IgG was eluted using 100mM glycine (pH 3.0) (Gibco BRL). Purified IgG concentration was quantified by a modified Bradford Assay (Bio-Rad).

2.5 – T. GONDII IgG AVIDITY ELISA

96-well microtitre plates (Greiner) were coated with 50µl of *T. gondii* antigen preparation (as previously described) at a concentration of 2 µg/ml in 0.1M carbonate/bicarbonate buffer (pH 9.6) overnight at 4°C. Following overnight incubation, the microtitre plates were washed 4 times in PBS/0.05% Tween-20 (PBS-T, pH 7.2) and blocked with 100µl per well of 5% goat serum (Gibco BRL) blocking solution in PBS-T and incubated at 37°C for 2 hours. Microtitre plates were then washed 4 times and 50µl of diluted serum (1:200 in PBS-T) was applied to the appropriate wells in duplicate and the plates were incubated at 4°C overnight. After 4 washes, 200µl of urea (Gibco BRL) was added to the wells at different concentration (0M, 2M, 6M in PBS-T) and incubated at room temperature for 1.5 hours. Following another 4 washes, 50µl of mouse anti-human IgG (Sigma) at a final dilution of 1:10000 in PBS-T was added to each well and the microtitre plates were incubated at room temperature for 2 hours. After 4 washes, 50µl of sheep anti-mouse F(ab')₂ (Jackson ImmunoResearch Laboratories Inc.) at a final dilution of 1:500 was added to

each well and the microtitre plates were incubated at room temperature for 1 hour. Following another 4 washes, 50 μ l of streptavidin-horseradish peroxidase conjugate (Gibco BRL) was added to each well at a final dilution of 1:1000 in PBS-T and incubated at room temperature for 30 minutes. After 4 more washes, 50 μ l of ABTS solution (prepared as previously described) was added to each well and microtitre plates were incubated at room temperature. OD₄₀₅ values were measured with an automatic ELISA plate reader (Titertek) after 10 minutes and 25 minutes of incubation at room temperature. The avidity of each sample was defined as the concentration of urea needed to dislodge 50% of the antibodies from the antigen in a given serum sample based upon extrapolation from a curve compared for each sample (versus OD₄₀₅ of no urea treatment).

2.6 – STATISTICAL ANALYSIS

In the text, mean OD₄₀₅ values are expressed as the mean $\pm$ the standard deviation. In the figures, error bars are expressed as standard error ([standard deviation]/square root [number of samples]). P-values are calculated using the Student's 2-tailed T-test. A p-value of 0.05 or lower was considered significant. Correlation coefficients (r) are determined by using the Pearson product moment coefficient calculation.

CHAPTER 3

RESULTS

3.1 – ELISA OPTIMIZATION EXPERIMENTS

3.1.1 – Direct Anti-Toxoplasma IgG ELISA

Optimization of the direct IgG ELISA was accomplished with the specific aim of demonstrating that the antigen preparation contained epitopes recognized by human serum. Positive and negative control sera (NCPS) were tested at serial dilutions using different concentrations of antigens. Figures 1A and 1B show that at optimal concentrations of antigen (1µg/ml) and serum (1/400) this assay could detect antibodies against *Toxoplasma*. The mean OD₄₀₅ for *Toxoplasma* positive sera was 0.961 ± 0.159 . The mean OD₄₀₅ for *Toxoplasma* negative sera was 0.119 ± 0.038 . In a modified version of this ELISA, the mAbs were shown to react with the crude whole tachyzoite antigen preparation (data not shown).

3.1.2 – Determination of Detection Limit for Antigen Capture ELISAs

Toxoplasma positive serum was simulated by spiking negative serum obtained from a healthy individual (IgM/IgG⁻) with crude whole tachyzoite antigen at concentrations ranging from 0µg/ml to 64µg/ml (two-fold serial dilutions). Spiked sera were then evaluated in both the first and second generation antigen detection ELISAs. The first generation assay detected a minimum of 8 µg/ml of *T. gondii* whole tachyzoite lysate (Figure 2A), while the second generation assay detected a minimum of 1-2 µg/ml of *T. gondii* whole tachyzoite lysate (Figure 2B).

3.2 – FIRST GENERATION ASSAY

3.2.1 – Determination of Assay “Cut-Off” Value

Sera obtained from 103 healthy Newfoundland adolescents found to be *Toxoplasma* IgG⁻ were used (13-17 years old, 1:1 gender ratio). The mean OD₄₀₅ value in the first generation antigen detection assay was 0.08 ± 0.06 . As a result, we established a cut-off value at an OD₄₀₅ equal to 0.200 (mean plus 2 standard deviations).

3.2.2 – Sensitivity of Assay

A preliminary evaluation of assay sensitivity used 19 specimens obtained from the NCPS serum archive (Figure 3). These specimens had been sent to the NCPS between 1985 and 1992 and were known to be IgG⁺ (n=19) and/or IgM⁺ (n=12) by “home grown” and poorly standardized NCPS ELISAs. No other information was available for these specimens. Using a 0.200 OD₄₀₅ cut-off value, 12/19 (63%) of these specimens were found to have circulating *Toxoplasma* antigen (mean OD₄₀₅: 0.528 ± 0.452) (Figure 3). Note that an OD₄₀₅ value of 0.200 in this assay corresponds roughly with the detection limit determined in the antigen-spiked serum (Figure 2A). By extrapolation from the spiked serum curve, it was possible to estimate the approximate concentrations of circulating antigen in these samples (Table 3). Upon expanded testing of this assay with a larger panel of better-characterized sera from the NCPS (n=100, 57 IgG⁺/IgM⁺, 24 IgG⁺/IgM⁻, 19 IgG⁻/IgM⁺), its sensitivity fell to 43%.

3.2.3 – Contribution of Individual Monoclonal Antibodies

A preliminary evaluation of 7 antigen positive sera (patients #1, 4, 9, 10, 15, 17, 19) using our antigen capture EIAs based upon individual mAbs revealed considerable heterogeneity (Figure 4). In some samples (patients #4, 10, 17), clone 45.15 appeared to contribute most of the reactivity observed. In other specimens, several other mAbs contributed significantly to the total observed reactivity (e.g.: clone 17.9 in patients #1 and #15). The contribution of the mAb (B3-90) directed against a 60 kDa internal antigen of the apical complex was consistently very low.

3.3 – SECOND GENERATION ANTIGEN DETECTION ELISA

3.3.1 – Determination of Assay “Cut-Off” Value

Sera obtained from healthy adults (OPHL, n=100) and from Newfoundland adolescents (n=103) found to be IgG⁻ were used. The mean OD₄₀₅ value for these sera in the second generation assay was 0.07 ± 0.05 for negative samples (n=203). As a result, we established a cut-off lower than the first generation assay at an OD₄₀₅ of 0.170 (mean of negative population plus 2 standard deviations).

3.3.2 – Assay Sensitivity

Sensitivity of the second generation assay was determined using 412 specimens obtained from the NCPS (n=124), LSPQ (n=7) and OPHL (n=281). These specimens (Table 4) were collected between 1988-1989 (n=114) and 1998-2000 (n=298) and were known to be IgG⁺ (n=269) and/or IgM⁺ (n=163) by the Platelia-IgG (OPHL, LSPQ) and the Toxo-IgM assays (OPHL, LSPQ) or by “home grown” IgM and IgG

ELISAs (NCPS). Using a 0.170 OD₄₀₅ cut-off value, 52% of these specimens (positives and equivocal: 213/412) were found to have circulating *Toxoplasma* antigen (mean OD₄₀₅: 0.612 ± 0.338 in 52% of the samples vs. 0.177 ± 0.319 for all 412 samples).

3.4 – EFFECT OF CIRCULATING IMMUNE COMPLEXES IN SERUM SAMPLES ON ANTIGEN DETECTION

3.4.1 – Circulating Immune Complexes

We hypothesized that circulating immune complexes (CIC) interfered with our ability to detect antigen. To test this hypothesis, we spiked 21 *Toxoplasma* antibody positive serum (7 IgG⁺/IgM⁺ samples, 7 IgG⁺/IgM⁻ samples, 7 IgG⁻/IgM⁺ samples) and 8 *Toxoplasma* antibody negative sera with 16 µg/ml of whole *Toxoplasma* tachyzoite lysate. The mean OD₄₀₅ in the spiked negative sera was 1.017 ± 0.069 suggesting good “recovery” of spiked antigen (Figure 5). In contrast, the formation of interfering CIC was readily observed in *Toxoplasma* antibody positive sera (Figure 6A-6C). Compared with a mean OD₄₀₅ change of 0.921 ± 0.068 in the antibody negative sera, the mean OD₄₀₅ change in the antibody positive sera was only 0.182 ± 0.061 . Among the antibody positive specimens, significant increases in OD₄₀₅ were only observed in the IgG⁺/IgM⁻ subgroup (0.320 ± 0.098 vs. 0.182 ± 0.061 ; $p=0.003$).

3.4.2 – Disruption of Immune Complexes using TCA

Several techniques have been described for the dissociation of immune complexes (IC) formed *in vitro* or *in vivo* so that bound antigen can be detected. Using 8% or 12% TCA to dissociate the IC formed *in vitro* in the spiked samples, we were able to

release and detect otherwise cryptic antigen. A representative experiment is shown in figure 7. Although 8% TCA-treatment revealed bound antigen successfully in some samples, 12% TCA-treatment permitted at least partial recovery of spiked antigen in almost all specimens.

3.5 – INFLUENCE OF ANTIBODY TITRE ON CAPACITY TO DETECT ANTIGEN

We proposed that antibody titre might also significantly influence our ability to detect circulating antigen. A total of 50 specimens were identified with IgM titres of 1:256 that differed widely in IgG titre (IgG = 1:1024, n=25 and IgG = 1:4096, n=25). As can be seen in figure 8, increasing IgG antibody titre was inversely related to antigen detection. Furthermore, (12%) TCA treatment resulted in much larger changes in OD₄₀₅ in the sera with low IgG titres compared to those with high titres (0.175 ± 0.022 vs. 0.056 ± 0.021 ; $p=0.05$).

3.6 – INFLUENCE OF ANTIBODY AVIDITY ON CAPACITY TO DETECT ANTIGEN

We demonstrated that IgG avidity also impeded our ability to detect antigen. IgG avidity was measured in 281 IgG⁺ sera obtained from the OPHL; 155 (55%) were also IgM⁺. Samples were not stratified according to IgM or IgG titre because this information was only available for a subset of sera. As can be seen from figure 9, there was a striking inverse relationship between IgG avidity and the change in OD₄₀₅ observed with TCA treatment ($r=0.57$, $p=0.0001$).

3.7 – APPLICATION OF TCA TO SECOND GENERATION ANTIGEN DETECTION ASSAY

Specimens from the OPHL were re-tested using either 8% TCA (n=245) or 12% TCA (n=254) to break CIC (Table 5A). The overall assay sensitivity increased to 96%. It is interesting that, if anything, TCA treatment lowered OD₄₀₅ values for IgM⁻/IgG⁻ samples (e.g.: No TCA: 0.075 ± 0.048 , vs. 8% TCA: 0.050 ± 0.033 or 12% TCA: 0.055 ± 0.053 ; p=0.0001 and p=0.05 respectively). In *Toxoplasma* antibody positive sera however, both 8% and 12% TCA significantly increased both the mean OD₄₀₅ and proportion of positives (e.g.: No TCA: 0.177 ± 0.319 vs. 8% TCA: 0.245 ± 0.139 or 12% TCA: 0.331 ± 0.287 ; p=0.0052 and p=0.0001 respectively) (Figure 10).

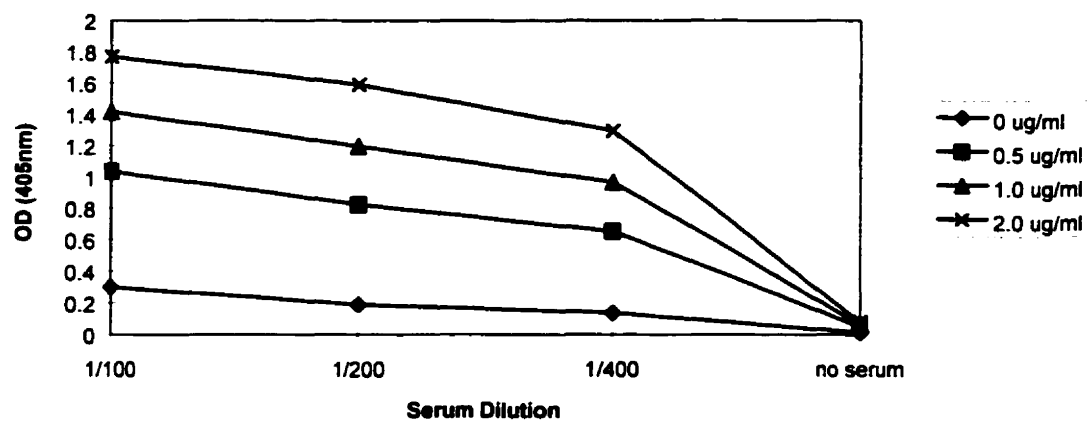
3.8 – ASSAY SPECIFICITY

The specificity of the 12% TCA-based assay was determined using 239 sera; 203 IgG⁻/IgM⁻ samples from the OPHL and Newfoundland serum banks and 36 (out of 50) specimens from subjects with other parasitic diseases obtained from the NCPS gold standard serum bank (36 *Toxoplasma* IgG⁻, 14 *Toxoplasma* IgG⁺) (Table 5B). The 12% TCA-based second generation assay was negative in all but 3/203 of the healthy IgG⁻/IgM⁻ controls for a specificity of 98.5%. In the subjects with other parasitic diseases, the specificity was slightly lower overall (90%) and lowest in specimens from subjects with malaria (n=6), a closer related apicomplexan parasite (40% positive).

3.9 – FIGURES & TABLES FOR RESULTS SECTION

Figure 1: Direct Anti-*Toxoplasma* IgG ELISA

[A] Serologically Positive Sera vs. Antigen Preparation



[B] Serologically Negative Sera vs. Antigen Preparation

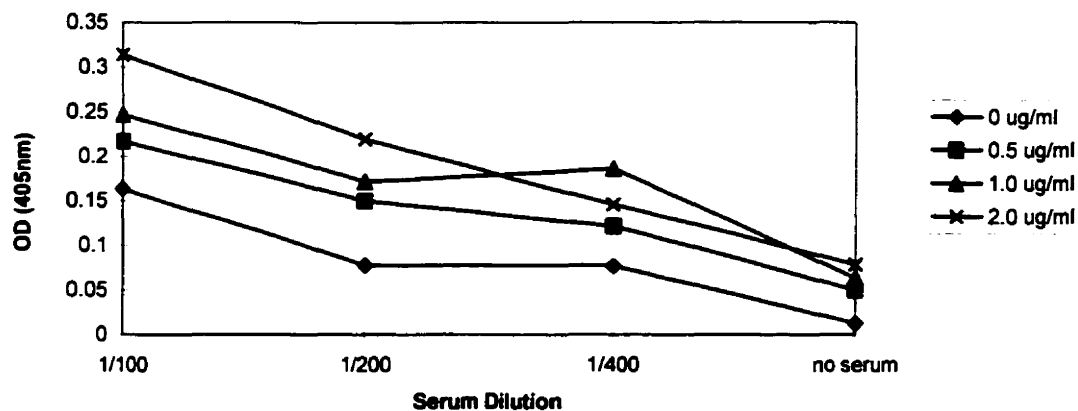
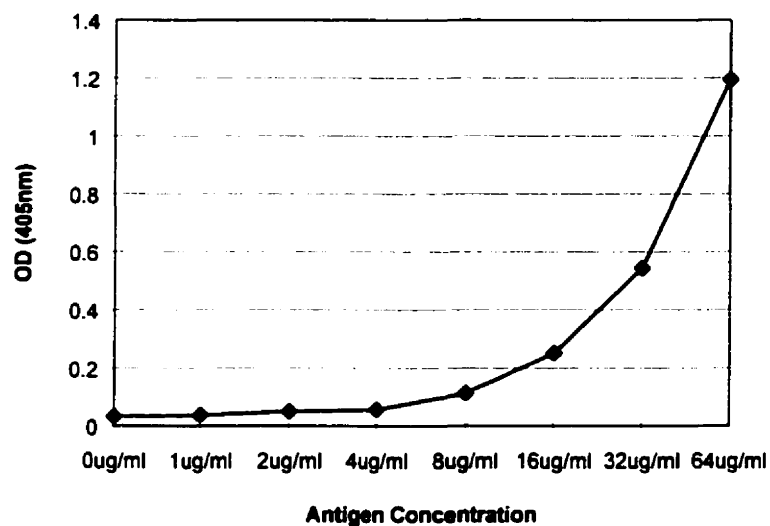


Figure 1: Direct IgG Capture Assay. The following antigen concentrations were measured: 2.0µg/ml (x), 1.0µg/ml (Δ), 0.5µg/ml (■) and 0µg/ml (◆). [A] Rises in absorbance readings positively correlate with increasing antigen concentrations and decreasing serum dilutions ($r=0.96$, mean OD_{405} : 0.961 ± 0.159). [B] Absorbance readings were lower with the negative sera (mean OD_{405} : 0.119 ± 0.038).

Figure 2: Determination of Antigen Detection Limit

[A] First Generation Assay



[B] Second Generation Assay

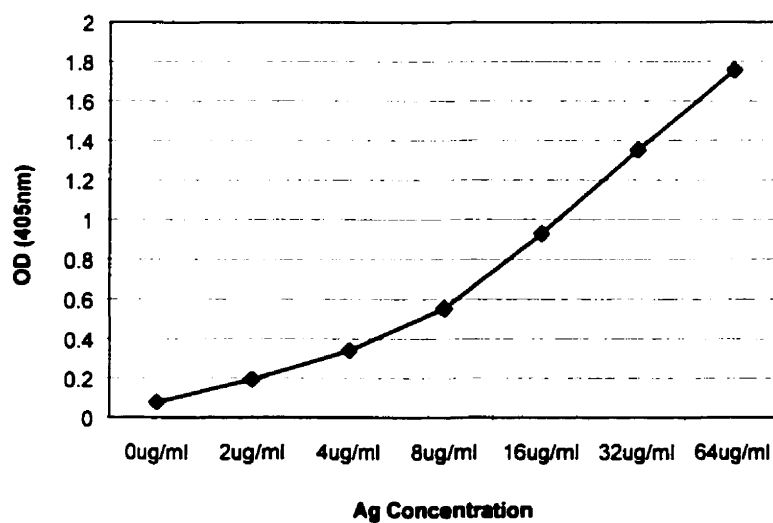


Figure 2: Determination of Antigen Detection Limit for First and Second Generation ELISAs. Negative sera spiked (♦) with varying antigen concentrations were tested for the presence of antigens. The pool of monoclonal antibodies was used to coat the plates. **[A]** Detection of a minimum of 8µg/ml of antigen was observed for the first generation assay. A signal to noise ratio of 36:1 was observed. **[B]** Detection of a minimum of 1-2µg/ml of antigen was observed for the second generation assay. A signal to noise ratio of 22:1 was observed.

Figure 3: Preliminary Sensitivity Evaluation of First Generation Assay

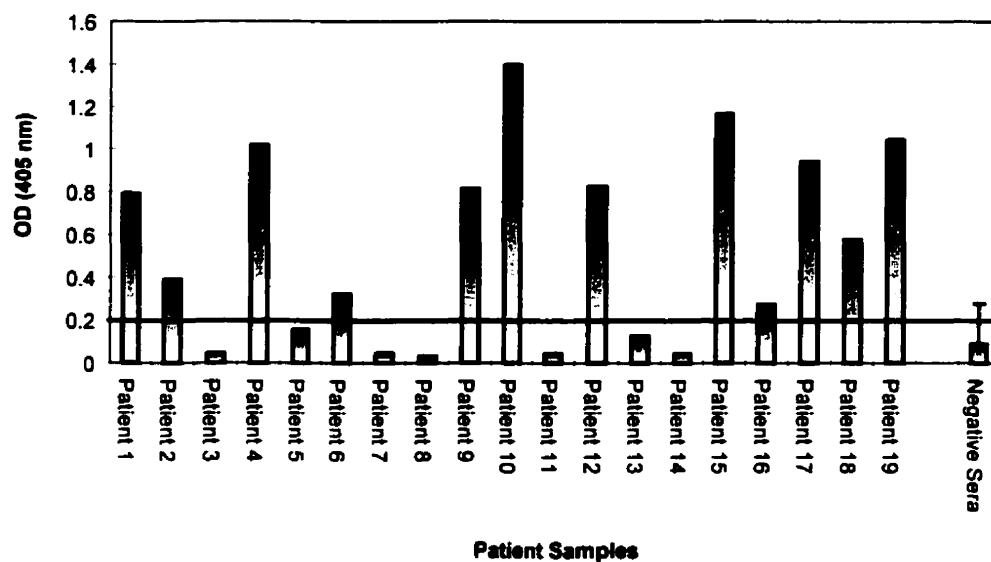


Figure 3: Preliminary Sensitivity Evaluation of First Generation Assay. 19 *Toxoplasma* positive sera were tested for circulating antigen using our antigen capture ELISA. In 63% of the samples, circulating antigens were detected (mean OD_{405} : 0.528 ± 0.452). The cut-off value, an OD_{405} of 0.200, based on the negative control ($n=103$, mean plus 2 standard deviations), was used as the basis of comparison with the positive sera.

Table 3: Approximate Antigen Concentration in Patient Samples

SPECIMEN	ANTIGEN CONCENTRATION
Patient 1	40µg/ml
Patient 2	25µg/ml
Patient 4	50µg/ml
Patient 5	12µg/ml
Patient 9	42µg/ml
Patient 10	80µg/ml
Patient 13	10µg/ml
Patient 15	64µg/ml
Patient 16	18µg/ml
Patient 17	46µg/ml
Patient 18	32µg/ml
Patient 19	60µg/ml

Table 3: Approximate Antigen Concentration in Patient Samples. OD₄₀₅ values from sera were compared to those of standard curve to extrapolate antigen concentration.

Figure 4: Detection of Circulating Antigens Using Individual Monoclonal Antibodies

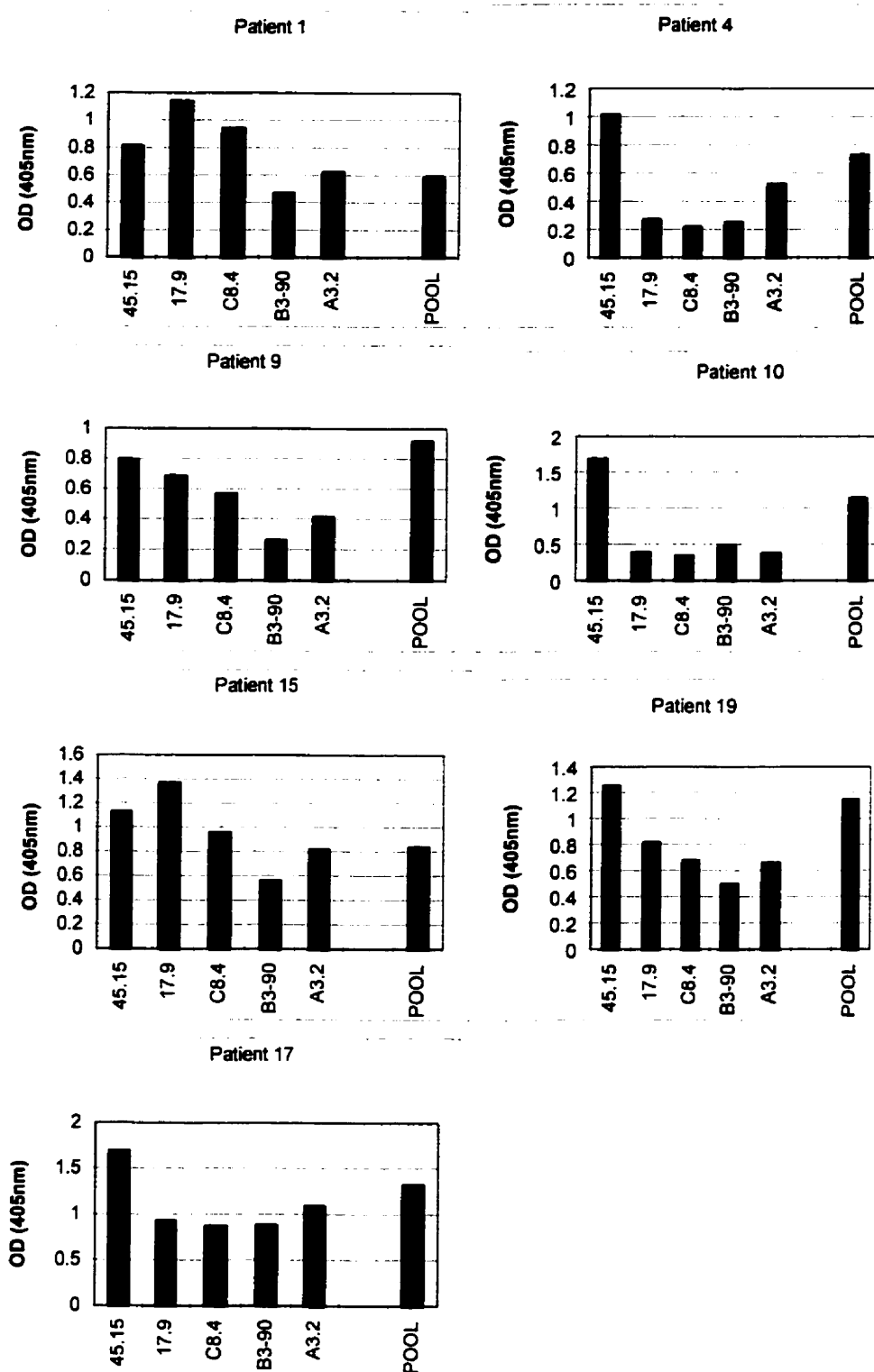


Figure 4: Detection of Circulating Antigens Using Individual Monoclonal Antibodies. Serum samples positive for circulating antigen were tested against each monoclonal antibody separately. The clone 45.15 detected circulating antigens in all samples while the clones 17.9 and C8.4 detected circulating antigens to a lesser extent. 7 samples are depicted separately with OD₄₀₅ values of the individual monoclonal antibodies compared to those of the pool.

Table 4: Sensitivity Evaluation of Second Generation Assay

NCPS	G+/M+	G+/M-	G-/M+	G-/M-
Total	57	48	19	10
(+)	29	24	10	1
(-)	17	22	6	8
(E)	11	2	3	1
% (+)	50-70%	50-54%	52-55%	1-2%
LSPQ	G+/M+	G+/M-	G-/M+	G-/M-
Total	3	0	4	0
(+)	1	0	2	0
(-)	1	0	2	0
(E)	1	0	0	0
% (+)	33-66%	0%	50%	0%
OPHL	G+/M+	G+/M-	G-/M+	G-/M-
Total	151	118	12	100
(+)	20	30	5	3
(-)	97	48	6	83
(E)	34	40	1	14
% (+)	13-36%	25-60%	42-60%	3-17%
NPHL	G+/M+	G+/M-	G-/M+	G-/M-
Total	0	0	0	103
(+)	0	0	0	0
(-)	0	0	0	103
(E)	0	0	0	0
% (+)	0%	0%	0%	0%
TOTAL	G+/M+	G+/M-	G-/M+	G-/M-
Total	211	166	35	213
(+)	50	54	17	4
(-)	115	70	14	194
(E)	46	42	4	15
% (+)	24-45%	33-68%	49-60%	2-8%

Table 4: Sensitivity Evaluation of Second Generation Assay. 412 *Toxoplasma* positive sera were tested for circulating antigen using our antigen capture ELISA. (+) refers to antigen positive samples, (-) refers to antigen negative samples, (E) refers to antigen equivocal samples. In 52% (213/412) of the samples, circulating antigens were detected (mean OD₄₀₅: 0.612 ± 0.338 in 52% of the samples vs. 0.177 ± 0.319 for all 412 samples). The cut-off value, an OD₄₀₅ of 0.170, based on the pool of negative sera (n=213, mean plus 2 standard deviations), was used as the basis of comparison with the positive sera.

Figure 5: Spiking of IgG⁺/IgM⁻ Specimens

Spiked Sera (IgG⁺/IgM⁻)

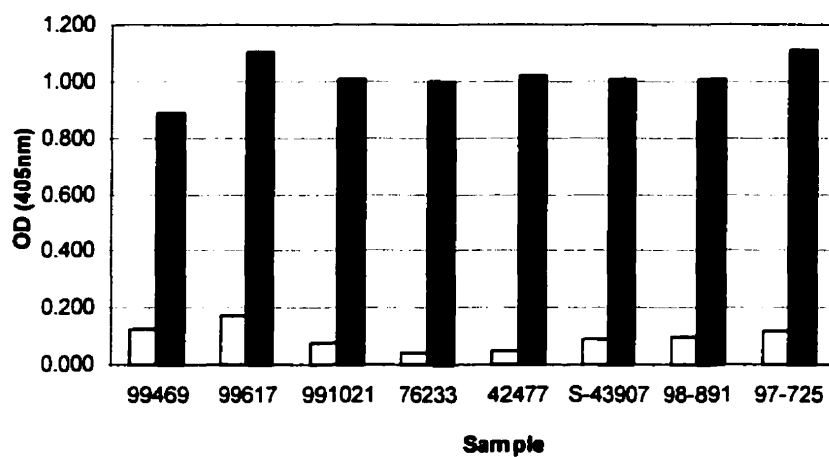
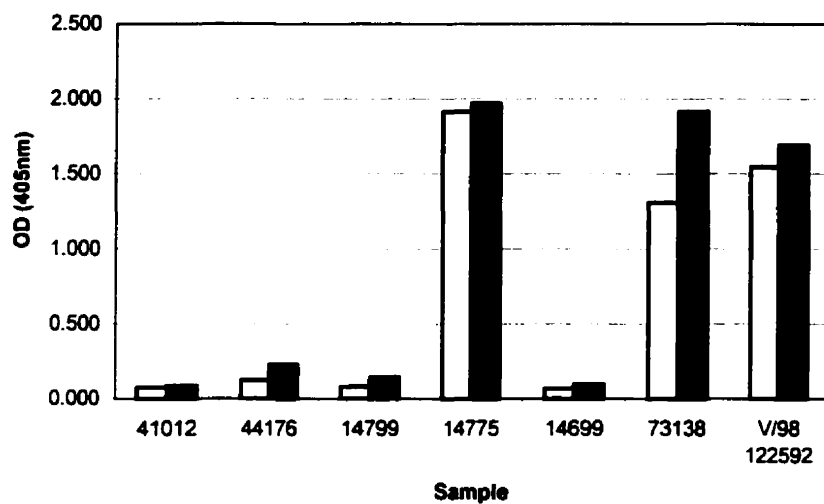
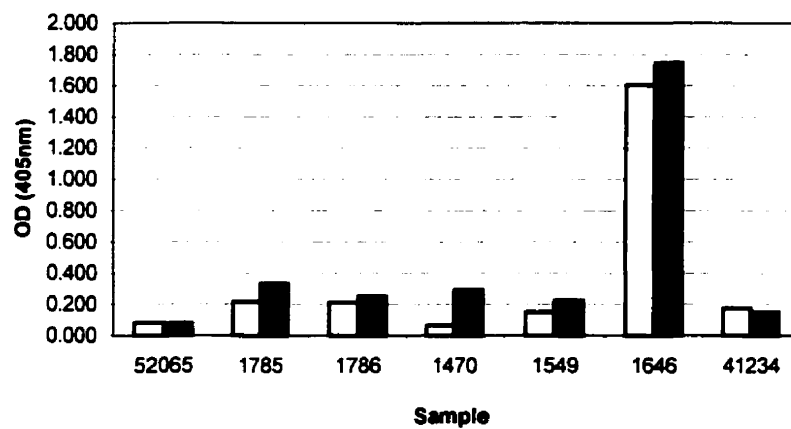


Figure 5: Spiking of IgG⁺/IgM⁻ Specimens. Each sample was spiked with 16µg/ml of *Toxoplasma gondii* whole tachyzoite lysate (black bars) and compared to its unspiked counterpart (white bars). CIC were not formed in the IgG⁺/IgM⁻ serological grouping. The mean OD₄₀₅ in the spiked negative sera was 1.017 ± 0.069.

Figure 6: Formation of Circulating Immune Complexes *in vitro***[A] Spiked Sera (IgG⁺/IgM⁺)****[B] Spiked Sera (IgG⁻/IgM⁺)**

[C] Spiked Sera (IgG⁺/IgM⁺)

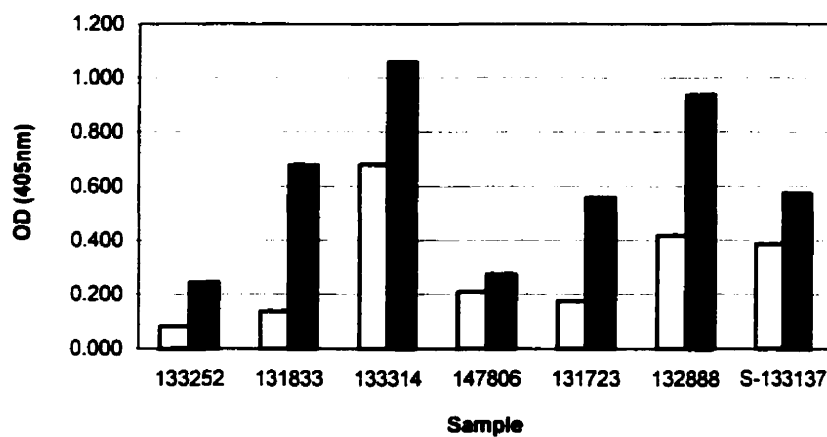


Figure 6: Formation of Circulating Immune Complexes *in vitro*. Samples (OPHL) were grouped according to serological status. [A] IgG⁺/IgM⁺, [B] IgG⁺/IgM⁺, [C] IgG⁺/IgM⁺. Each sample was spiked with 16µg/ml of *Toxoplasma gondii* whole tachyzoite lysate (black bars) and compared to its unspiked counterpart (white bars). CIC were observed when black bars and white bars were at similar levels.

Figure 7: *In vitro* Dissociation of Circulating Immune Complexes

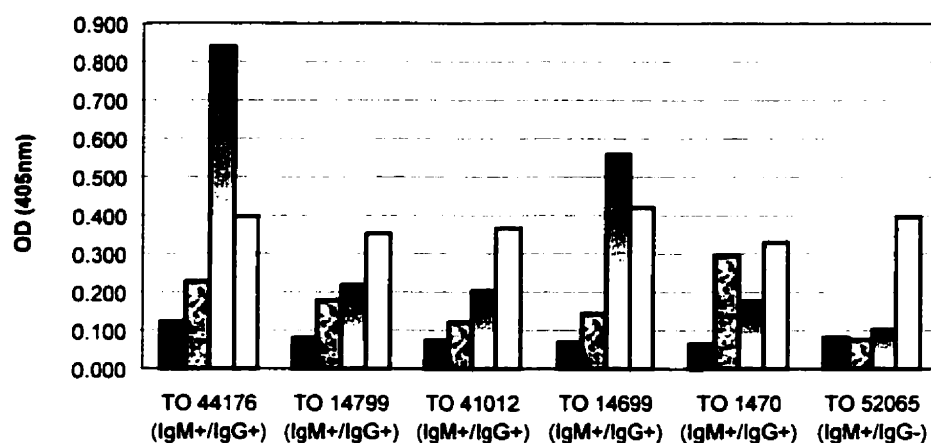
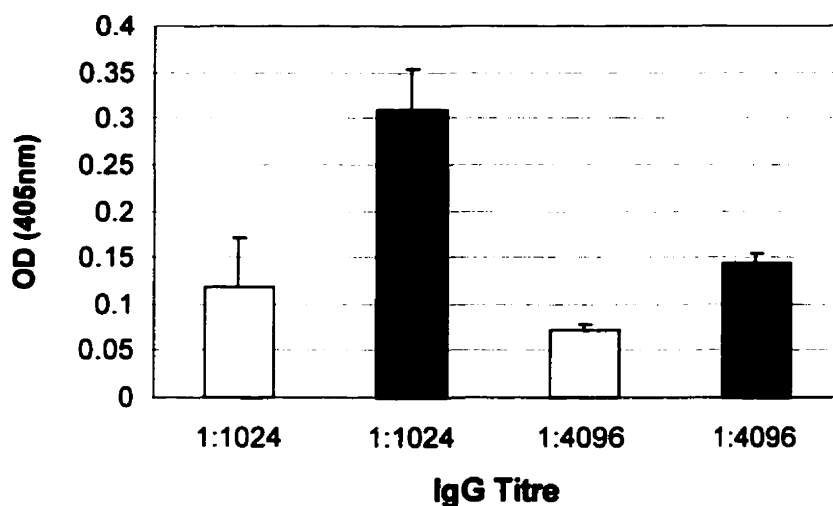


Figure 7: *In vitro* Dissociation of Circulating Immune Complexes. Samples in which CIC were formed were treated with 8% TCA (grey gradient bars) and 12% TCA (white bars). OD₄₀₅ readings were compared to unspiked (black bars) and spiked (dotted bars) (16µg/ml of *Toxoplasma gondii* whole tachyzoite lysate) versions of the same sample. The use of TCA clearly dissociates CIC as seen by an increase in OD₄₀₅ values in all samples.

Figure 8: Effect of Antibody Titre on Antigen Detection and Destruction of Circulating Immune Complexes in unspiked sera

[A] IgM 1:256 – 12% TCA



[B] IgM 1:256 – 8% TCA

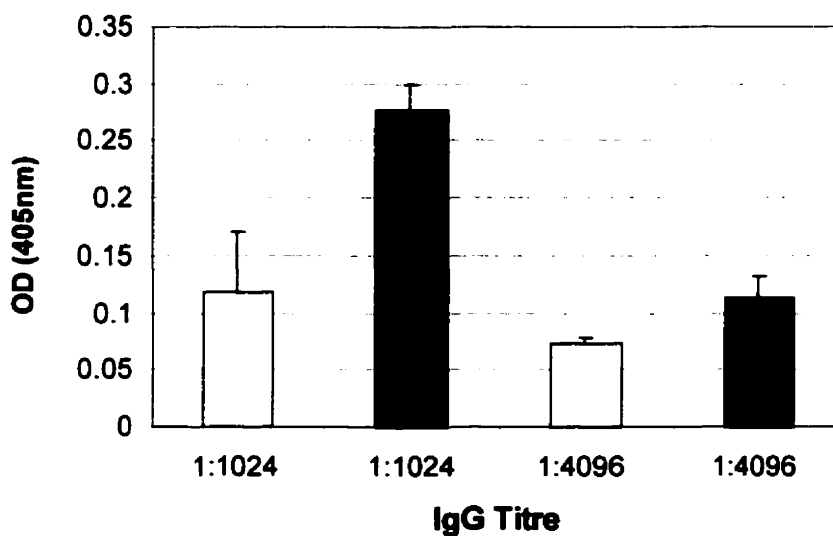


Figure 8: Effect of Antibody Titre on Antigen Detection and Destruction of Circulating Immune Complexes in Unspiked Sera. In a representative experiment (n=50), samples were grouped according to serological status. With IgM titre held constant (1:256), samples were then divided according to IgG titres: higher (1:4096, n=25) and lower (1:1024, n=25). With Increasing IgG titre, antigen detection dropped. Upon the use of either 12% TCA [A] or 8 % TCA [B] (black bars), the level of antigen detection increased by 3 times with the same trend remaining.

Figure 9: Effect of Antibody Avidity on Antigen Detection

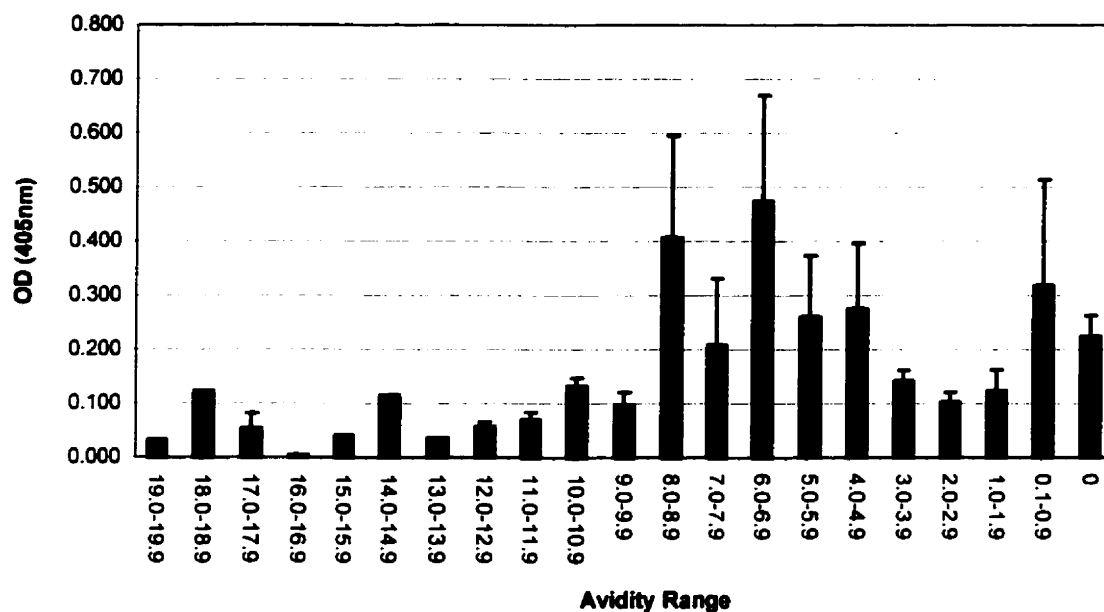


Figure 9: Effect of Antibody Avidity on Antigen Detection. 281 samples were tested for IgG antibody avidity. An arbitrary range of avidity units (0-19.9) was established. High avidity antibodies ranged from 9.0 to 19.9 avidity units (n=75), medium avidity antibodies ranged from 4.0 to 8.9 avidity units (n=124) low avidity antibodies ranged from 0.1 to 3.9 avidity units (n=61) and no avidity antibodies located at 0 avidity units (n=21). Increasing IgG antibody avidity correlated with decreasing ability to detect antigens ($r=0.57$, $p=0.0001$).

Figure 10: The Use of TCA to Improve Assay Sensitivity

Antigen Detection vs. TCA Treatment

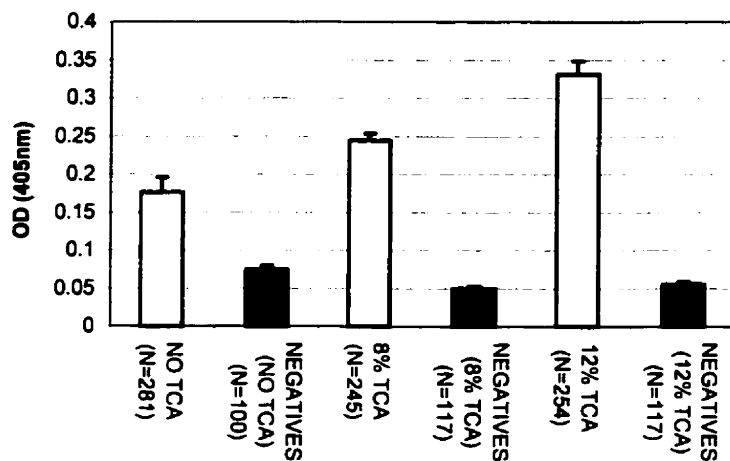


Figure 10: The Use of TCA to Improve Assay Sensitivity. Samples (OPHL) were grouped according to serological status. White bars indicate serologically positive samples and black bars indicate serologically negative samples. The use of 8% TCA was significant in increasing antigen detection sensitivity ($p=0.005$, $n=245$) and in decreasing background noise in negative samples ($p=0.0001$, $n=117$). The use of 12% TCA was also significant in increasing antigen detection sensitivity ($p=0.0001$, $n=254$) and in decreasing background noise in negative samples ($p=0.05$, $n=117$).

Table 5: Summary of Samples Tested

[A] Assay Sensitivity

SEROLOGY	0% TCA	8% TCA	12% TCA
G+/M+	13-36% (n=151)	83-85% (n=123)	89-95% (n=132)
G+/M-	25-60% (n=118)	97-100% (n=114)	100% (n=114)
G-/M+	42-50% (n=12)	100% (n=8)	100% (n=8)
TOTAL	20-46% (n=281)	90-93% (n=245)	94-96% (n=254)

[B] Assay Specificity

SOURCE	SPECIMENS	% IgG NEGATIVE	% Ag NEGATIVE
Newfoundland	n=103	100%	100%
OPHL	n=100	100%	97%
Other Parasitic Diseases (NCPS)	Amebiasis (n=5)	80%	80%
	Cysticercosis (n=6)	100%	100%
	Filariasis (n=10)	90%	100%
	Hydatid Disease (n=10)	50%	100%
	Malaria (n=6)	17%	60%
	Schistosomiasis (n=9)	89%	89%
	Trichinosis (n=4)	75%	100%
TOTAL	n=253 (n=239)	85% _{..}	97% _{..}

Table 5: [A] Assay Sensitivity. All samples tested (OPHL) to determine assay sensitivity are summarized. Optimal assay conditions suggest the use of 12% TCA (sensitivity=96%, n=254). **[B] Assay Specificity.** Specimens were collected from the Newfoundland and OPHL serum banks and from the NCPS gold-standard serum bank. Specimens were assayed for anti-*Toxoplasma* antibodies using the direct anti-*Toxoplasma* IgG ELISA (85% of the specimens were IgG⁺). Assay specificity was measured using the 12% TCA-based antigen capture assay. 97% of specimens were antigen negative. Note that only 36 samples (IgG⁺) from the 50 specimens collected from other parasitic diseases were used to calculate actual assay specificity.

CHAPTER 4

DISCUSSION

Toxoplasma gondii causes a wide range of clinical presentations in humans; manifestations range from asymptomatic illness to death, with the latter being observed mainly in a small subset of newborn children and in immunocompromised patients (1, 2, 3). In the majority of cases, the diagnosis of acute toxoplasmosis is confirmed by serology (4, 19). However, the production of specific antibodies may be delayed or impaired, especially in immunodeficient patients (275). Furthermore, serologic tests are not always able to discriminate between recent and past infection, as is needed in the cases of suspected congenital toxoplasmosis (4, 19, 275). An alternative to serology is the detection of parasite specific antigens, which may be present in the blood stream or body fluids during active infection.

The detection of circulating *T. gondii* antigens in human serum was first reported in 1977 (262). Although several investigators initially suggested that the simultaneous detection of *Toxoplasma*-specific IgM antibodies and circulating antigens would be a better indication of acute infection than the detection of IgM antibodies alone, the antigen detection efforts reported to date have not demonstrated reasonable sensitivity in human samples (61-83, 262-275).

Before entering into a detailed discussion of our findings, it would be appropriate to review the strengths and weaknesses of the clinical specimens that we used to test our assay. These specimens have two major strengths. First, we used a large number of samples (n=675) as compared with the other published studies (61-83, 262-275). Second, most of the samples that we used were characterized by state-of-the-art

standardized EIAs in public health laboratories (OPHL, LSPQ, NCPS). Unfortunately, there are four obvious weaknesses associated with these sera. First, only limited clinical and demographic information was available for these specimens. With respect to the OPHL, LSPQ and NCPS samples, these were originally tested in routine screening protocols or in the context of suspected clinical illness. Therefore, it can be assumed that the majority of these samples were obtained from asymptomatic pregnant women, immunocompromised subjects with possible reactivated infection (e.g.: HIV patients and patients receiving immunosuppressive drugs) and immunocompetent subjects with symptomatic toxoplasmosis (e.g.: flu- or mononucleosis-like symptoms). Second, although the vast majority of the samples were stored undisturbed at -20°C for less than two years in the OPHL, LSPQ and NCPS, the storage conditions and period of storage varied for the remaining samples. Third, the sera from St. John's, Newfoundland were obtained from asymptomatic adolescents and were tested in a "home-grown" NCPS *Toxoplasma* IgG-ELISA (all negative). These samples are not "ideal negative controls" but were most useful with respect to testing the assay's specificity. Gold-standard negative control sera (e.g.: pregnant women and other adults) will need to be tested in order to further evaluate assay sensitivity. Finally, the NCPS samples used to determine the specificity of the assay (i.e.: samples from other parasitic diseases, $n=50$) were not fully characterized (i.e.: positive only for known parasitic disease but not exhaustively tested for other parasitic diseases). Despite the inherent weaknesses in this serum bank and although false positives and negatives can occur in EIA testing, the large number of samples

we tested should permit us to make a cautious first estimate of both assay sensitivity and specificity with reasonable confidence.

The most striking observation of the data presented is the overall high sensitivity of the assay (96%, n=254) (Table 5A). In previous reports, no study has demonstrated an overall sensitivity above 75%. To date, there have been no major clinical studies examining the potential use of antigen detection in toxoplasmosis as in HIV, EBV and CMV infections, where information about circulating antigens is more useful than antibody data with regard to the diagnosis of these infections. The biology of the parasite, the nature of the specimens and the novel aspects of our assay (i.e.: dissociation of CIC) are factors that can explain our results.

Once *T. gondii* infects its host (i.e.: humans), sporozoites released from the cysts infect a wide variety of tissues and rapidly undergo endodyogeny to form tachyzoites. The acute phase of toxoplasmosis is characterized by the production of IgM (and IgA) antibodies. During this period of high parasite burden, the tachyzoites can disseminate to other tissues in the body and infect muscles, fibroblasts, the liver and nerves. As the infection is increasingly controlled by the host's cell-mediated immune response, tachyzoite proliferation slows, causing a chronic infection (i.e.: the parasite is never eliminated). Asexual reproduction in these tissues is much slower than at the original site and the parasite develops into bradyzoites and tissue cysts. This chronic phase is characterized by the presence of specific IgG antibodies. With respect to humans, *T. gondii* causes a life long infection. Although circulating antibodies are

used as evidence of infection, the humoral response plays a minor role in the control of toxoplasmosis. As for most intracellular parasites, cell-mediated immunity and particularly interferon-gamma (IFN- γ), produced by Th1 cells, is crucial for an effective defense against this infection: mice treated by anti-IFN- γ or deficient for the IFN- γ gene are highly susceptible to toxoplasmosis (300, 301). Furthermore, it has been demonstrated in mouse models that Th2 cytokines tend to promote parasite growth (302). The activity of parasite replication is likely to be inversely proportional to this cell-mediated immune response and as such, the infection is never truly quiescent. This claim is supported in HIV patients by the fact that there is a high incidence of *Toxoplasma* infections associated with these immunodeficient individuals. We can anticipate that the likelihood that parasite antigens will be detectable in the circulation would be greatest during acute toxoplasmosis (i.e.: IgG⁻/IgM⁺ infections). However, the biology of the parasite and its relationship with the host's immune response make it plausible that IgG⁺/IgM⁻ patients have detectable levels of circulating antigen. This may be particularly true in pregnant women and in cases of reactivated disease in immunocompromised patients.

In our work, we were not studying sera from immunologically "normal" human specimens. The majority of the specimens that we tested was obtained from pregnant women, subjects with reactivated disease (e.g.: HIV patients, patients receiving immunosuppressive therapy) and acutely infected individuals. Each of these groups would be predicted to have less control of *Toxoplasma*. First, in pregnant women, a pregnancy that is successful represents a relatively immunocompromised state (303).

Maternal acceptance of the fetal-allograft is in part due to suppression of cell-mediated cytotoxicity (304). This is accomplished by the release of immunosuppressive cytokines such as IL-4, IL-6 and IL-10 (305). This pattern of cytokine secretion, characteristic of a relative increase in Th2-associated immunity and decreased Th1 immunity, could facilitate the rupture of tissue cysts and the dissemination of the parasite in the prospective mother (305). Second, in immunocompromised patients, concomitant infection (e.g.: with HIV) or administration of immunosuppressive drugs (e.g.: used in organ transplant procedures) suppresses the immune response's ability to control the parasite, allowing for rapid parasite multiplication. Finally, with regard to acutely infected individuals, they would have less control of a *Toxoplasma* infection for a number of reasons: [1] during acute infection there is a high parasite burden, [2] the immune response has not successfully inhibited parasite propagation to the point where tachyzoites encyst into bradyzoites, and [3] only low affinity IgM antibodies are present to bind with the parasite. Since it is unlikely that patients remain *Toxoplasma* antigen positive for life (due to cell-mediated control of *T. gondii*), a state of immunosuppression in a large number of samples could have dramatically increased our ability to detect antigen. Therefore, the apparent high sensitivity of our assay may be an artifact of our samples. However, when studying samples from other parasitic diseases not originally collected for *Toxoplasma* screening or diagnosis (n=50), 14 sera were antibody positive, while 35% of these sera were antigen positive. This sensitivity falls well within the range of previous studies. Further evaluation of immunologically "normal" samples, will provide more insight into the true sensitivity of the assay.

The third and final reason why our sensitivity is so high can be explained by the dissociation of CIC (i.e.: the circulating antigen was already complexed with antibody and was therefore not detectable in our assay). Previous studies that dealt with antigen detection (e.g.: *Schistosoma mansoni*, *Onchocerca volvulus*, *Wucheria bancrofti*, *Brugia malayi*), have resolved low sensitivities by dissociating CIC in sera with the use of agents such as TCA (260, 307) and EDTA (173, 187). We first demonstrated conclusively the formation of CIC by spiking *Toxoplasma* antibody positive and negative specimens. Most of the *Toxoplasma* antibody positive sera had the capacity to make the spiked antigen “disappear” (Figure 6). The mean OD₄₀₅ change in *Toxoplasma* antigen spiked negative sera (ΔOD_{405} : 0.921 ± 0.068) was more than 4-fold higher than that in *Toxoplasma* antigen spiked antibody positive sera (ΔOD_{405} : 0.182 ± 0.061). Overall, these data strongly suggest: [1] that antibody/antigen complexes could be formed *in vitro* with “excess” antibody, [2] that antibody/antigen complexes interfered with our antigen detection assay, and [3] that the presence of the antibody/antigen complexes in our specimens was virtually certain.

These results allowed us to further optimize our assay. Following the *in vitro* demonstrations of immune complex formation with spiked serum samples, we used TCA to dissociate the CIC. Without CIC dissociation, the sensitivity of the assay was 52% (n=412), comparable to other studies conducted using animal and human specimens. However, the use of 12% TCA increased the assay sensitivity to 96% (n=254).

Using these serum samples and the novel dissociation of CIC, we have designed a qualitative and semi-quantitative antigen capture ELISA (the second generation assay). The quantitative nature of the assay can be improved by running serial dilutions of samples in order to establish an antigen titre. Although our assay is only semi-quantative, when we compared the mean OD₄₀₅ change between acute infections (IgG⁻/IgM⁺) and chronic infections (IgG⁺/IgM⁻) after 12% TCA treatment (i.e.: after CIC dissociation), we observed that the mean OD₄₀₅ change was greater in the acute infections (ΔOD_{405} : 0.250 ± 0.06 vs. 0.185 ± 0.123 ; $p=0.001$). This could be attributed to the immunosuppressed state of the patient and/or the constant “ticking-over” of the parasite. Previous studies that examined antigen detection in these patients failed to report high sensitivities (52, 55, 57, 94, 98, 105, 108-111, 114, 117). In these patients, the suppressed immune response would cause tachyzoite proliferation to commence, allowing for an increase of parasite burden in the host. This would theoretically enhance the ability to detect circulating antigen with a higher sensitivity. The inability to detect antigen at high levels in these previous studies may be due to the fact that the authors did not attempt to dissociate CIC. Had we used specimens obtained solely from immunocompetent males, asymptomatic cases or from banks of serum that were not originally collected for screening purposes, then the sensitivity of our assay might have been lower.

Previous studies have described CIC in toxoplasmosis (308, 309) but only one other study in humans, conducted by Lindenschmidt, has significantly dealt with the dissociation of CIC (310). However after dissociation of the CIC, circulating antigens

were isolated in only 30% (n=42) of patients in this study (310). This low sensitivity may be due to the following: [1] the CIC were dissociated using 3M NaSCN and not 12% TCA, [2] the samples were poorly characterized (i.e.: the samples were obtained from patients with lymphadenopathy, presumed to be due to acute-phase toxoplasmosis), [3] the IgM titres were determined by using an IgM-IFAT and not an IgM-ELISA and [4] the antigen detection ELISA did not use mAbs and was not performed in a sandwich format. It was then proposed that CIC should be measured in conjunction with serological testing, rather than circulating antigens, as the CIC test provides more useful information about the activity of a *Toxoplasma* infection (308, 309). However, the major flaw with this proposal is that CIC may be found in a relatively high proportion of clinically healthy individuals with or without *Toxoplasma* infection. We believe that the detection of circulating antigens (not CIC) provides the most useful information with regard to the activity of a *Toxoplasma* infection. We demonstrated that 12% TCA-treatment permitted at least partial recovery of spiked antigen in almost all *Toxoplasma* antibody positive specimens (Figure 7). Furthermore, the use of TCA not only had a significant impact on increasing antigen detection ($p=0.0001$), it was also able to significantly decrease the number of false positives and the background noise in negative samples ($p=0.05$) (Figure 10). However, the true sensitivity of our assay will eventually be determined by: [1] testing large numbers of samples with defined clinical sera, [2] longitudinal studies conducted on IgG⁺/IgM⁻ samples from pregnant women and/or immunocompromised hosts (e.g.: pre and post immune suppression therapy) and [3] longitudinal studies conducted on samples from *Toxoplasma* outbreaks (e.g.: Victoria,

British Columbia, Canada) (41). These peculiarities of our sera and our novel dissociation of CIC might explain why previous studies have failed in the past to detect comparable levels of circulating antigens.

To date, our limited specificity testing has been conducted using 103 Newfoundland adolescents, 100 IgG/IgM specimens from the OPHL and 36 individuals with various parasitic diseases. Assay specificity was 97% (n=239). Based on the samples used in this study, this high specificity indicates that there are minimal numbers of false-positives with the use of our assay and low amounts of cross-reactions with other parasitic diseases (Table 5B). Assay specificity was lower with samples from other parasitic diseases (especially a related apicomplexan parasite – *Plasmodium spp.*) for a number of reasons: [1] False positives: other related-parasitic diseases may contain cross-reacting epitopes and test positive, [2] Age of infection: these samples were obtained most probably from patients in endemic areas and may have been exposed to *Toxoplasma* at a young age, [3] Antibody Titre: patients who were *Toxoplasma* antibody positive may not necessarily be infected. Further studies will need to be conducted on samples known to cross-react in antibody detection EIAs causing false positives (e.g.: samples obtained from cancer, lupus and arthritis patients). However, unlike antibody detection EIAs, we do not expect that the nature of these samples will influence the result of the antigen detection assay. This might prove to be a major strength of our antigen detection assay.

During the development of our assay, our data raised the possibility that both antibody titre and avidity might influence our results. Although only qualitative antibody data were available for most of the serum samples, IgM and IgG titres were recorded for some. A subset of these latter specimens was used to test the hypothesis that antibody titres influenced our ability to detect antigen *ex-vivo*. In *Toxoplasma* infection, IgG antibodies are produced slowly after IgM production. The IgG titres increase with time until the infection becomes quiescent. At this point, there is a basal amount of tachyzoites being produced and the majority of the parasites are encysted in tissue as bradyzoites. This suggests that with increasing antibody titre, there is a lower amount of circulating parasite and the ability to successfully detect antigen should decrease. We demonstrated that increasing antibody titres were inversely related to our ability to detect antigen (Figure 8). Furthermore, a strong association was observed between antibody titre and the mean OD₄₀₅ change upon 12% TCA treatment in the sera with low IgG titres as compared to those with high titres (ΔOD_{405} : 0.175 ± 0.022 vs. 0.056 ± 0.021 ; $p=0.05$) suggesting that we were able to detect more antigen in the samples with lower antibody titres (i.e.: higher amount of parasite production) after CIC dissociation. In the lower titre samples, there will be fewer host-serum derived antibodies to interfere with the antigen detection ELISA than in the higher titre samples, which may also explain the difference in OD₄₀₅ changes.

IgG avidity was then studied as a possible confounder in our ability to detect circulating antigens. Several investigators have shown that the avidity of anti-

Toxoplasma antibodies increased quite slowly over a period of 3-4 months (88-90). Our prediction was that sera with lower avidity antibodies would be obtained earlier in infection (hence more likely to have circulating antigen) and that these low avidity antibodies might also be more likely to release bound antigen in response to TCA treatment. We observed a striking correlation between IgG avidity and the mean change in OD₄₀₅ observed with TCA treatment ($r=0.57$, $p=0.0001$) (Figure 9), which suggests that antigen is more readily detectable upon TCA treatment in samples with lower antibody avidity (i.e.: lower antibody titre and higher amounts of parasite production). However, the nature of our specimens precludes any final conclusions because IgM⁺ and low avidity sera are likely to be observed earlier in infection.

Currently, the presence of *Toxoplasma*-specific IgG antibodies indicates infection at some point, but the level of IgG reactivity is not indicative of how recently the individual was infected. Determining the timing of infection in pregnant women and whether or not the infection has reactivated in immunocompromised patients are extremely important so that the appropriate measures can be taken to limit the risk of damage to the fetus and patient respectively. Currently the sole non-invasive method capable of discriminating between pregnancies at high versus low risk is IgM testing (1, 4, 19, 275, 306). Unfortunately, problems with the specificity of commercial tests have resulted in the presentation of erroneous information to the physician and his or her patient, which have interfered with decisions related to clinical management (1, 4, 19, 275, 306). Furthermore, IgM antibodies have been detected as long as 2 years after the initial infection in some people (2). Standard serologic tests are of little value

in the recognition of reactivated disease. In designing our *Toxoplasma* antigen detection assay, we had hoped that we could discriminate between acute and chronic infections. This proved not to be the case however since our assay was 95% sensitive in IgG⁺/IgM⁻ sera and 100% in IgM⁺ sera. Therefore, in the assay's present state, it cannot replace the current serological diagnostic tests.

In order to use this assay for the diagnosis of reactivated disease in immunocompromised patients and in congenital infection, strain (42, 50) and/or stage (311, 312) specific antigens may need to be recognized. These antigens could potentially have great diagnostic use in the differentiation between acute and chronic infections based on their appearance in the biological life-cycle of the parasite. For example, in immunocompromised patients, a drop in the overall immune response to infection would tend to correlate with increasing multiplication of parasites (i.e.: increasing levels of parasite burden) and release of tachyzoites from tissue cysts. The use of a tachyzoite specific antigen may allow for the rapid diagnosis of this reactivated infection. Previous studies have shown that *T. gondii* comprises three clonal lineages, types I, II and III (50) and that the majority of human infections (66%) are caused by type II (50). Congenital infections seem to be caused mainly by type I and type II, while type III is observed mainly in animals (50). Furthermore, it has been demonstrated that there are major antigenic differences between these strains, detectable by Western blotting and parasite plaque reduction assays (311, 312). The possibility that the parasite genotype influences the severity of human disease is supported by differences in the pathogenicity of parasite strains in animal

models. Type II strains produce high cyst burdens in mice (313-315) and are prone to reactivate in experimentally immunocompromised mice (316). These traits may increase the risk of reactivation of chronic infection, leading to toxoplasmic encephalitis in immunocompromised humans (e.g.: AIDS patients). Type I strains (e.g.: RH strain) are extremely virulent in mice and cause significantly higher levels of parasitemia (317, 318). It has been observed in humans that such high parasitemia (caused by types I and II) may increase the risk of transplacental transmission or severity of infection in the developing fetus. *Toxoplasma* stage specific antigens have been demonstrated for all three major morphologic life-cycle stages: tachyzoite (311, 312), bradyzoite (311, 312) and oocyst (311, 312, 319, 320). The antigens located in the P30 cluster of the immunoreactive proteins between molecular weights of 24 to 34 kDa (311) may be of particular interest since the mAbs 17.9 and A3.2, used in our assay, were raised against 31 kDa and 34 kDa dense granule proteins respectively. For example, the identification of a type II strain-specific tachyzoite antigen in a pregnant woman could plausibly be of real value in evaluating the risk of congenital infection.

Although we have designed a highly sensitive and specific assay, what remains to be done is the following: [1] increased testing with immunologically “normal” samples and longitudinal serum samples (e.g.: patients pre and post immunosuppressive therapy) to determine the true sensitivity and specificity of the assay, [2] further studies using individual mAbs (as opposed to a pool of mAbs) with fully characterized specimens will be required to understand the heterogeneity revealed by

the mAbs in our preliminary data (Figure 4) and [3] the use of individual mAbs should be investigated to see if particular antigens are associated with one of the three *T. gondii* genotypes and to see if they are associated with particular clinical profiles (e.g.: pregnant women, immunocompromised hosts, acutely infected patients). Depending on the results of this expanded work, *Toxoplasma* antigen testing may yet prove to be valuable in the clinical situations of particular interest. However, our sensitivity and specificity data to date suggest that *Toxoplasma* antigen detection may have some significant advantage over routine antibody EIAs in some clinical settings (e.g.: acutely infected patients). In conclusion, although this study was conducted using samples based solely on serological status, the data suggests that further evaluation with more defined sera is warranted to confirm the usefulness of our antigen detection test as a diagnostic tool for monitoring *T. gondii* infection.

REFERENCES

REFERENCES

- 1) **Frenkel, J.K.** 1990. Toxoplasmosis in human beings. *JAVMA*. **196** (2): 240-248.
- 2) **Frenkel, J. K.** 1985. Toxoplasmosis. *Pediatr. Clin. N. America*. **32**(4): 917-932.
- 3) **Remington, J. S.** 1990. The tragedy of toxoplasmosis. *Pediatr. Infect. Dis. J.* **9**(10): 762-763.
- 4) **Wilson, M., J. S. Remington, C. Clavet, G. Varney, C. Press and D. Ware.** 1997. Evaluation of six commercial kits for the detection of human immunoglobulin M antibodies to *Toxoplasma gondii*. *J. Clin. Microbiol.* **35**(12): 3112-3115.
- 5) **Hafid, J., S. Tran Manh Sung, H. Raberin, Z. Y. Akono, B. Pozzetto and M. Janna.** 1995. Detection of circulating antigens of *Toxoplasma gondii* in human infection. *Am. J. Trop. Med. Hyg.* **52** (4): 336-339.
- 6) **Nicolle, M. M. C. and L. Manceaux.** 1908. Sur une infection a corps de Leishman (ou organismes voisins) du gondii. *C. R. Acad. Sci (Paris)*. **147**: 763-766.
- 7) **Splendore, A.** 1908. Un nuovo protozoa parrassita de'conigli. Inconfrato nelle lesioni anatomiche d'una malattia che ricorda in molti punti il kala-azar dell'uomo. *Rev. Soc. Scient. Sao Paulo*. **3**: 109-112.
- 8) **Wolf, A., D. Cowen and B. Paige.** 1939. Human toxoplasmosis: occurrence in infants as an encephalomyelitis – verification by transmission to animals. *Science*. **89**: 226-227.
- 9) **Sabin, A. B.** 1942. Toxoplasmosis: A recently recognized disease of human beings. *Adv. Pediatr.* **1**: 1-53.
- 10) **Pinkerton, H. and D. Weinman.** 1940. *Toxoplasma* infection in man. *Arch. Pathol.* **30**: 374-392.
- 11) **Sabin, A. B. and H. A. Feldman.** 1948. Dyes as microchemical indicators of a new immunity phenomenon affecting a protozoan parasite (*Toxoplasma*). *Science*. **108**: 660-663.
- 12) **Walls K. W., S. L. Bullock, D. K. English.** 1977. Use of the enzyme-linked immunosorbent assay (ELISA) and its microadaptation for the serodiagnosis of toxoplasmosis. *J. Clin. Microbiol.* **5**(3):273-277.
- 13) **Lin T. M., S. P. Halbert, G. R. O'Connor.** 1980. Standardized quantitative enzyme-linked immunoassay for antibodies to *Toxoplasma gondii*. *J. Clin. Microbiol.* **11**(6): 675-681.

- 14) Naot, Y. and J. S. Remington. 1980. An enzyme-linked immunosorbent assay for the detection of IgM antibodies to *Toxoplasma gondii*: use for diagnosis of acute acquired toxoplasmosis. J. Infect. Dis. **142**(5): 757-766.
- 15) Frenkel, J. K. Toxoplasmosis: parasite life cycle, pathology and immunology. In: Hammond D.M., Long P. L. eds. The Coccidia. *Eimeria*, *Isospora*, *Toxoplasma* and related genera. Baltimore, MD: University Park Press, 1973: 343-410.
- 16) Dubey, J. P., N. L. Miller and J. K. Frenkel. 1970. The *Toxoplasma gondii* oocyst from cat feces. J. Exp. Med. **132**(4): 636-662.
- 17) Dubey, J. P., D.S. Lindsay and C. A. Speer. 1998. Structures of *Toxoplasma gondii* tachyzoites, bradyzoites, and sporozoites and biology and development of tissue cysts. Clin. Microbiol. Rev. **11**(2): 267-299.
- 18) Jacobs, L., J. S. Remington and N. L. Melton. 1960. The resistance of the encysted form of *Toxoplasma gondii*. J. Parasitol. **46**: 11.
- 19) Wilson, M., and J. B. McAuley. 1991. Laboratory diagnosis of toxoplasmosis. Clin. Lab. Med. **11**(4): 923-939.
- 20) McCabe, R. E., and J. K. Frenkel. 1988. Toxoplasmosis: The time has come. N. Engl. J. Med. **318**(5): 313-315.
- 21) Ruskin, J. and J. S. Remington. 1968. Immunity and intracellular infection: resistance to bacteria in mice infected with a protozoa. Science. **160**: 72.
- 22) Gentry, L. O. and J. S. Remington. 1971. Resistance against *Cryptococcus* conferred by intracellular bacteria and protozoa. J. Infect. Dis. **123**(1): 22-31.
- 23) Hibbs, J. B., L. H. Lambert and J. S. Remington. 1971. Resistance to murine tumors conferred by chronic infection with intracellular protozoa, *Toxoplasma gondii* and *Besnoitia jellisoni*. J. Infect. Dis. **124**(6): 587-592.
- 24) Hibbs, J. B., L. H. Lambert and J. S. Remington. 1972. Possible role of macrophage mediated nonspecific cytotoxicity in tumor resistance. Nature (New Biol.). **235**(54): 48-50.
- 25) Hibbs, J. B., L. H. Lambert and J. S. Remington. 1972. Control of carcinogenesis: possible role for the activated macrophage. Science. **177**(53): 998-1000.
- 26) Frenkel, J. K. 1967. Adoptive immunity to intracellular infection. J. Immunol. **98**: 1309.
- 27) Shirahatz, T., N. Shimizu and N. Suzuki. 1976. Effects of immune lymphocyte products and serum antibody on the multiplication of *Toxoplasma* in murine peritoneal macrophages. Z. Parasitenkd. **49**(1): 11-23.
- 28) Jones, T. C. 1974. Macrophages and intracellular parasitism. J. Reticuloendothel. Soc. **15**(5): 439-450.

- 29) **Krahenbuhl, J. L., J. Ruskin and J. S. Remington.** 1972. The use of killed vaccines in immunization against an intracellular parasite: *Toxoplasma gondii*. *J. Immunol.* **108(2)**: 425-431.
- 30) **Hatizi, A. and F. Z. Modabber.** 1978. Effect of cyclophosphamide on *Toxoplasma gondii* infection: reversal of the effect by passive immunization. *Clin Exp. Immunol.* **33(3)**: 389-394.
- 31) **Remington, J. S.** 1974. Toxoplasmosis in the adult. *Bull. NY Acad. Med.* **50(2)**: 211-217.
- 32) **Toussaint, D. and J. J. Vanderhaeghen.** 1975. Ocular toxoplasmosis, triennial herpes zoster and pulmonary tuberculosis in a patient with Hodgkin's disease. *Ophthalmologica.* **171(3)**: 237-243.
- 33) **Friedman, A. H.** 1984. The retinal lesions of the acquired immunodeficiency syndrome. *Trans. Am. Ophthalmol. Soc.* **82**: 447-491.
- 34) **Launais, B., G. Laurent, S. Berchkroun, J. J. Moulin, J. Pris, P. Bec and J. Monnier.** 1983. Manifestations meningo-encephaliques et chorioretiniennes de la toxoplasmose chez un malade immunodeprimé. *Sem. Hop. Paris* **1(1)**: 40.
- 35) **Perke, D. W.** 1986. Diffuse toxoplasmic retinochoroiditis in a patient with AIDS. *Arch. Ophthalmol.* **104(4)**: 571-575.
- 36) **Weiss, A., C. E. Margo, D. K. Ledford, R. F. Lockey and J. H. Brinser.** 1986. Toxoplasmic retinochoroiditis as an initial manifestation of the acquired immunodeficiency syndrome. *Am. J. Ophthalmol.* **101(2)**: 248-249.
- 37) **Frenkel, J. K. and A. Escajadillo.** 1987. Cyst rupture as a pathogenic mechanism of toxoplasmic encephalitis. *Am J. Trop. Med. Hyg.* **36(3)**: 517-522.
- 38) **Remington, J. S., R. McLeod and G. Desmonts.** Toxoplasmosis. In: Remington, J. S., Klein, J. O. eds. *Infectious diseases of the fetus and newborn infant*. Philadelphia: WB Saunders, 1995: 140-267.
- 39) **Bertoli, F., M. Espino, J. R. Arosemena, J. L. Fishback and J. K. Frenkel.** 1995. A spectrum in the pathology of toxoplasmosis in patients with AIDS. *Arch. Pathol. Lab. Med.* **119(3)**: 214-224.
- 40) **Luft, B. J. and J. S. Remington.** Toxoplasmosis of the central nervous system. In Remington, J. S., Swartz, M. N. eds. *Current Clinical Topics in Infectious Diseases*. New York: McGraw-Hill, 1985:315.
- 41) **Bell, A., J. Isaac-Renton, A. King, L. Martinez, D. Roscoe, D. Werker, S. Eng, T. Johnstone, R. Stanwick, et al.** 1995. Outbreak of toxoplasmosis associated with municipal drinking water—British Columbia. The British Columbia Toxoplasmosis Team. *Can. Commun. Dis. Rep.* **21(18)**: 161-163.
- 42) **Sibley, L. D. and J. C. Boothroyd.** 1992. Virulent strains of *Toxoplasma gondii* comprise a single clonal lineage. *Nature.* **359(6390)**: 82-85.

- 43) **Ruskin, J. and J. S. Remington.** 1976. Toxoplasmosis in the compromised host. *Am. Intern. Med.* **84**(2): 193-199.
- 44) **Luft, B. J., F. K. Conley, J. S. Remington, M. Laverdiere, K. F. Wagner, J. F. Levine, P. C. Craven, D. A. Strandberg, T. M. File, F. Meunier-Carpentier.** 1983. Outbreak of CNS toxoplasmosis in Western Europe and North America. *Lancet.* **1**(8328): 781-784.
- 45) **Nolla-Sallas, J. C. Ricart, L. D'Ohlaberringue, F. Gali and J. Lamorca.** 1987. Hydrocepholis: an unusual CT presentation of cerebral toxoplasmosis in a patient with acquired immunodeficiency syndrome. *Eur. Neurol.* **27**(2): 130-132.
- 46) **Milligan, S. A., M. S. Katz, P. C. Craven, D. A. Strandberg, I. J. Russell and R. A. Becker.** 1984. Toxoplasmosis presenting as parhypopituitarism in a patient with acquired immunodeficiency syndrome. *Am. J. Med.* **77**(4): 760-764.
- 47) **Foulon, W., A. Naessens, T. Mahler, M. De Waele, L. De Catte and F. De Meuter.** 1990. Prenatal diagnosis of congenital toxoplasmosis. *Obstetrics & Gynecology.* **76**(5): 769-772.
- 48) **Ades, A. E.** 1991. Evaluating the sensitivity and predictive value of tests of recent infection: toxoplasmosis in pregnancy. *Epidemiol. Infect.* **107**(3): 527-535.
- 49) **Wilson, C. B., J. S. Remington, Stagno, S. and D. W. Reynolds.** 1980. Development of adverse sequelae in children born with subclinical congenital *Toxoplasma* infection. *Pediatrics.* **66**(5): 767-774.
- 50) **Howe, D. K. and D. Sibley.** 1995. *Toxoplasma gondii* comprises three clonal lineages: correlation of parasite genotype with human disease. *J. Infect. Dis.* **172**(6): 1561-1566.
- 51) **Acebes, M. V., B. Diez, J. A. Garcia-Rodriguez, P. Viens and R. Cisterna.** 1994. Detection of circulating antigens in the diagnosis of acute toxoplasmosis. *Am. J. Trop. Med. Hyg.* **51**(4): 506-511.
- 52) **Hassan, M. M., S. A. Mansour, M. Atta, M. M. Shalaby, M. A. Seksaka and A. Awad.** 1997. The importance of detecting circulating *Toxoplasma* antigens in human cases. *J. Egypt. Soc. Parasitol.* **27**(1): 27-34.
- 53) **Derouin, F., M. C. Mazon and J. F. Garin.** 1987. Comparative study of tissue culture and mouse inoculative methods for the demonstration of *Toxoplasma gondii*. *J. Clin. Microbiol.* **25**: 1597-1600.
- 54) **Hughes, H. P., L. Hudson and D. C. Fleck.** 1986. *In vitro* culture of *Toxoplasma gondii* in primary and established cell lines. *Int. J. Parasitol.* **16**: 317-322.
- 55) **Hoffin, J. M. and J. S. Remington.** 1985. Tissue culture isolation of *Toxoplasma* from blood of a patient with AIDS. *Arch. Intern. Med.* **145**: 925-926.

- 56) **Remington, J. S. and G. Desmonts.** Toxoplasmosis, in Remington, J. S., Klein, J. O. eds.: *Infectious Diseases of the Fetus and Newborn Infant*, 2nd ed. Philadelphia, Saunders, 1983, 143-263.
- 57) **Derouin, F., C. Sarfati, B. Beauvais, M. Iliou, L. Dehen and M. Lariviere.** 1989. Laboratory diagnosis of pulmonary toxoplasmosis in patients with acquired immunodeficiency syndrome. *J. Clin. Microbiol.* **27**: 1661-1663.
- 58) **Holliman, R. E., J. Johnson and M. Burke.** 1990. False-negative dye test findings in a case of fatal toxoplasmosis associated with cardiac transplantation. *J. Infect. Dis.* **21**: 185.
- 59) **Welch, P. C., H. Masur, T. C. Jones and J. S. Remington.** 1981. Serologic diagnosis of acute lymphadenopathic toxoplasmosis. *J. Infect. Dis.* **142**(2): 256-264.
- 60) **Malhotra, V. L., Y. Bharadwaj, A. Lakshmy, H. Kapur and K. Prakash.** 1991. Comparison of enzyme linked immunosorbent assay and indirect haemagglutination test in serologic diagnosis of toxoplasmosis. *J. Commun. Dis.* **23**(2): 154-156.
- 61) **al-Meshari, A. A., M. N. Chowdhury, S. K. Chattopadhyay and S. K. De Silva.** 1989. Screening for toxoplasmosis in pregnancy. *Int. J. Gynaecol. Obstet.* **29**(1): 39-45.
- 62) **Conteras, M., H. Schenone, P. Salinas, L. Sandoval, A. Rojas, F. Villarroel and F. Solis.** 1996. Seroepidemiology of human toxoplasmosis in Chile. *Rev. Inst. Med. Trop. Sao Paulo.* **38**(6): 431-435.
- 63) **Lappin, M. R. and C. C. Powell.** 1991. Comparison of latex agglutination, indirect hemagglutination and ELISA techniques for the detection of *Toxoplasma gondii*-specific antibodies in the serum of cats. *J. Vet. Intern. Med.* **5**(5): 299-301.
- 64) **Dubey, J. P., P. Thulliez, R. M. Weigel, C. D. Andrews, P. Lind and E. C. Powell.** 1995. Sensitivity and specificity of various serologic tests for detection of *Toxoplasma gondii* infection in naturally infected sows. *Am. J. Vet. Res.* **56**(8): 1030-1036.
- 65) **Fulton, J. D., J. L. Turk.** 1958. Direct agglutination test for *Toxoplasma gondii*. *Lancet.* **11**: 1068-1069.
- 66) **Desmonts, G. and J. S. Remington.** 1980. Direct agglutination test for diagnosis of *toxoplasma* infection: method for increasing sensitivity and specificity. *J. Clin. Microbiol.* **11**: 562-568.
- 67) **Johnson, J., K. Duffy, L. New, R. E. Holliman, B. S. Chessum and D. G. Fleck.** 1989. Direct agglutination test and other assays for measuring antibodies to *Toxoplasma gondii*. *J. Clin. Pathol.* **42**(5): 536-541.
- 68) **Janitschke, K., W. Busch and C. Kellershofen.** 1988. Direct agglutination as a tool for *Toxoplasma* control in pregnancy. *Immun. Infekt.* **16**(5): 189-191.

- 69) **Brooks, R. G., R. E. McCabe and J. S. Remington.** 1987. Role of serology in the diagnosis of toxoplasmic lymphadenopathy. *Rev. Infect. Dis.* **9(5)**: 1055-1062.
- 70) **Suzuki, Y. and J. S. Remington.** 1990. Importance of membrane-bound antigens of *Toxoplasma gondii* and their fixation for serodiagnosis of toxoplasmic encephalitis in patients with acquired immunodeficiency syndrome. *J. Clin. Microbiol.* **28(10)**: 2354-2356.
- 71) **Payne, R. A., J. M. Francis and W. Kwantes.** 1984. Comparison of a latex agglutination test with other serological tests for the measurement of antibodies to *Toxoplasma gondii*. *J. Clin. Pathol.* **37(11)**: 1293-1297.
- 72) **Wreghitt, T. G., J. J. Gray and A. H. Balfour.** 1986. Problems with the serological diagnosis of *Toxoplasma gondii* infections in heart transplant recipients. *J. Clin. Pathol.* **39(10)**: 1135-1139.
- 73) **Ise, Y., T. Iida, K. Sato, T. Suzuki and K. Shimada.** 1981. Studies on non specific reactions in the *toxoplasma* latex agglutination test. *Jpn. J. Parasitol.* **30**: 579-585.
- 74) **Sutehall, G. M. and T. G. Wreghitt.** 1989. False positive latex tests by ELISA for *Toxoplasma* IgG. *J. Clin. Pathol.* **42(2)**: 204-205.
- 75) **Holliman, R. E., J. Johnson, K. Duffy and L. New.** 1989. Discrepant *Toxoplasma* latex agglutination test results. *J. Clin. Pathol.* **42(2)**: 200-203.
- 76) **Wood, J. K., A. H. Balfour, E. S. Prestage, I. Noel and T. G. Wreghitt.** 1988. False positive results in the Toxoreagent test for *Toxoplasma gondii* in immunocompetent patients. *J. Clin. Pathol.* **41(10)**: 1135-1136.
- 77) **Guruz, A. Y., U. Z. Ok and M. Korkmaz.** 1996. Assessment of latex indirect agglutination test (Toxolater Fumouze) for the detection of *Toxoplasma* specific antibodies in human sera in Turkey. *J. Egypt. Soc. Parasitol.* **26(2)**: 367-374.
- 78) **Holliman, R. E., K. F. Barker and J. D. Johnson.** 1990. Selective antenatal screening for toxoplasmosis and the latex agglutination test. *Epidemiol. Infect.* **105(2)**: 409-414.
- 79) **el-Gamal, R. L., A. M. Farghaly, M. E. Abdel-Fattah and A. M. el-Ridi.** 1989. Comparative study between latex agglutination and indirect immunofluorescent antibody tests in diagnosis of toxoplasmosis. *J. Egypt. Soc. Parasitol.* **19(2)**: 471-476.
- 80) **Payne, R. A., J. M. Francis and W. Kwantes.** 1984. Comparison of a latex agglutination test with other serological tests for the measurement of antibodies to *Toxoplasma gondii*. *J. Clin. Pathol.* **37(11)**: 1293-1297.
- 81) **Walton, B. C., B. M. Benchoff and W. H. Brooks.** 1966. Comparison of indirect fluorescent antibody test and methylene blue dye test for detection of antibodies to *Toxoplasma gondii*. *Am. J. Trop. Med. Hyg.* **15**: 149-152.

- 82) **Radulovic, S., L. Videnovic, B. Jokovic, D. Dordevic, R. Lalic, D. Pokorni, P. Mijuskovic and M. Cirkovic.** 1990. Use of the ELISA immunoenzyme test and indirect immunofluorescence in the diagnosis of a toxoplasmosis epidemic. *Vojnosanit. Pregl.* **47(4)**: 276-279.
- 83) **Naot, Y., E. V. Barnett and J. S. Remington.** 1981. Method for avoiding false positive results occurring in immunoglobulin M enzyme-linked immunosorbent assays due to the presence of both rheumatoid factor and antinuclear antibodies. *J. Clin. Microbiol.* **14(1)**: 73-78.
- 84) **Budzko, D. B., L. Tyler and D. Armstrong.** 1989. Fc receptors on the surface of *Toxoplasma gondii* trophozoites: a confounding factor in testing for anti-*Toxoplasma* antibodies by indirect immunofluorescence. *J. Clin Microbiol.* **27(5)**: 959-961.
- 85) **Wilson, M., D. A. Ware and K. W. Walls.** 1987. Evaluation of commercial serodiagnostic kits for toxoplasmosis. *J. Clin. Microbiol.* **25(12)**: 2262-2265.
- 86) **Liesenfeld, O., C. Press, J. G. Montoya, R. Gill, J. L. Isaac-Renton, K. Hedman and J. S. Remington.** 1997. False-positive results in immunoglobulin M (IgM) *Toxoplasma* antibody tests and importance of confirmatory testing: the Platelia Toxo IgM test. *J. Clin. Microbiol.* **35(1)**: 174-178.
- 87) **Fuccillo, D. A., D. L. Madden, N. Tzan and J. L. Sever.** 1987. Difficulties associated with serological diagnosis of *Toxoplasma gondii* infections. *Diagn. Clin. Immunol.* **5(1)**: 8-13.
- 88) **Liesenfeld, O., J. G. Montoya, S. Kinney, C. Press and J. S. Remington.** 2001. Effect of testing for IgG avidity in the diagnosis of *Toxoplasma gondii* infection in pregnant women: experience in a US reference laboratory. *J. Infect. Dis.* **183(8)**: 1248-1253.
- 89) **Auer, H., A. Vander-Mose, O. Picher, J. Walochnik and H. Aspöck.** 2000. Clinical and diagnostic relevance of the *Toxoplasma* IgG avidity test in the serological surveillance of pregnant women in Austria. *Parasitol. Res.* **86(12)**: 965-970.
- 90) **Holliman, R. E., R. Raymond, N. Renton and J. D. Johnson.** 1994. The diagnosis of toxoplasmosis using IgG avidity. *Epidemiol. Infect.* **112(2)**: 399-408.
- 91) **Lindenschmidt, E. G.** 1986. Enzyme-linked immunosorbent assay for detection of soluble *Toxoplasma gondii* antigenic component p35000 by enzyme-linked antigen immunosorbent assay. *J. Clin. Microbiol.* **24(6)**: 1045-1049.
- 92) **Decoster, A., F. Darcy and A. Capron.** 1988. Recognition of *Toxoplasma gondii* excreted and secreted antigens by human sera from acquired and congenital toxoplasmosis: identification of markers of acute and chronic infection. *Clin. Exp. Immunol.* **73(3)**: 376-382.

- 93) **Potasman, I., F. G. Araujo, G. Desmonts and J. S. Remington.** 1986. Analysis of *Toxoplasma gondii* antigens recognized by human sera obtained before and after acute infection. *J. Infect. Dis.* **154**(4): 650-657.
- 94) **Hassl, A., H. Aspöck and H. Flamm.** 1988. Circulating antigen of *Toxoplasma gondii* in patients with AIDS: Significance of detection and structural properties. *Zentralbl. Bakteriol. Mikrobiol. Hyg. [A].* **270**(1-2): 302-309.
- 95) **Huskinson, J., P. Thulliez and J. S. Remington.** 1990. *Toxoplasma* antigens recognized by human immunoglobulin A antibodies. *J. Clin. Microbiol.* **28**(12): 2632-2636.
- 96) **Robert-Gangneux, F., V. Commerce, C. Tourte-Scafer and J. Dupouy-Camet.** 1999. Performance of a Western Blot assay to compare mother and newborn anti-*Toxoplasma* antibodies for early neonatal diagnosis of congenital toxoplasmosis. *Eur. J. Clin. Microbiol. Infect. Dis.* **18**(9): 648-654.
- 97) **Menard, D., L. Paris and M. Danis.** 1999. Immunoblot applied to the diagnosis of congenital toxoplasmosis. *Pathol. Biol. (Paris).* **47**(8): 797-804.
- 98) **Weiss, L. M., S. A. Udem, H. tanowitz and M. Wittner.** 1988. Western blot analysis of the antibody response of patients with AIDS and *Toxoplasma* encephalitis: antigenic diversity among *Toxoplasma* strains. *J. Infect. Dis.* **157**(1): 7-13.
- 99) **Partanen, P., H. J. Turunen, R. T. A. Paasivuo and P. O. Leinikki.** 1984. Immunoblot analysis of *Toxoplasma gondii* antigens by human immunoglobulins G, M and A antibodies at different stages of infection. *J. Clin. Microbiol.* **20**(1): 133-135.
- 100) **Potasman, I., F. G. Araujo, G. Desmonts and J. S. Remington.** 1986. Analysis of *Toxoplasma gondii* antigens recognized by human sera obtained before and after acute infection. *J. Infect. Dis.* **154**(4): 650-657.
- 101) **Verhofstede, C., P. van Gelder and M. Rabaey.** 1988. The infection-stage-related IgG response to *Toxoplasma gondii* studied by immunoblotting. *Parasitol. Res.* **74**(6): 516-520.
- 102) **Moir, I. J., M. M. Davidson and D. O. Ho-Yen.** 1991. Comparison of IgG antibody profiles by immunoblotting in patients with acute and previous *Toxoplasma gondii* infection. *J. Clin. Pathol.* **44**(7): 569-572.
- 103) **Yamamoto, Y. I., J. R. Mineo, C. S. Meneghisse, A. C. Guimaraes and M. Kawarabayahi.** 1998. Detection in human sera of IgG, IgM and IgA to excreted/secreted antigens from *Toxoplasma gondii* by use of dot-ELISA and immunoblot assay. *Ann. Trop. Med. Parasitol.* **92**(1): 23-30.
- 104) **Angel, S., E. Maero, J. C. Blanco, V. Pzsenny, C. Zala, R. Gonzalez, H. Perelmutter and J. C. Garberi.** 1992. Early diagnosis of toxoplasmic encephalitis in AIDS patients by dot blot hybridization analysis. *J. Clin. Microbiol.* **30**: 3286-3287.

- 105) **Cristina, N., H. Pelloux, C. Goulhot, J. P. Brion, P. Leclercq and P. Ambroise-Thomas.** 1993. Detection of *Toxoplasma gondii* in AIDS patients by the polymerase chain reaction. *Infection*. **21**: 150-153.
- 106) **Gross, U., A. Roggenkamp, K. Janitschke and J. Heesemann.** 1992. Improved sensitivity of the polymerase chain reaction for detection of *Toxoplasma gondii* in biological and human clinical specimens. *Eur. J. Clin. Microbiol. Infect. Dis.* **11**: 33-39.
- 107) **Johnson, J. D., P. D. Butcher, D. Savva and D. E. Holliman.** 1993. Application of the polymerase chain reaction to the diagnosis of human toxoplasmosis. *J. Infect. Dis.* **16**: 147-158.
- 108) **Lebech, M., A.-M. Lebech, S. Nelsing, J. Vuust, L. Mathiesen and E. Petersen.** 1992. Detection of *Toxoplasma gondii* DNA by polymerase chain reaction in cerebrospinal fluid from AIDS patients with cerebral toxoplasmosis. *J. Infect. Dis.* **165**: 982-983.
- 109) **Ostergaard, L. A., A. K. Nielsen and F. T. Black.** 1993. DNA amplification on cerebrospinal fluid for diagnosis of cerebral toxoplasmosis among HIV-positive patients with signs or symptoms of neurological disease. *Scand. J. Infect. Dis.* **25**: 227-237.
- 110) **Parmley, S. F., F. D. Goebel and J. S. Remington.** 1992. Detection of *Toxoplasma gondii* in cerebrospinal fluid from AIDS patients by polymerase chain reaction. *J. Clin. Microbiol.* **30**: 3000-3002.
- 111) **Schoondermark-van de Ven, E., J. Galama, C. Kraaijeveld, J. van Druten, J. Meuwissen and W. Melchers.** 1993. Value of the polymerase chain reaction for the detection of *Toxoplasma gondii* in cerebrospinal fluid from patients with AIDS. *Clin. Infect. Dis.* **16**: 661-666.
- 112) **Van de Ven, E., W. Melchers, J. Galama, W. Camps and J. Meuwissen.** 1991. Identification of *Toxoplasma gondii* infections by B1 gene amplification. *J. Clin. Microbiol.* **29**: 2120-2124.
- 113) **Verhofstede, C., S. Reniers, R. Colebunders, F. van Wanseele and J. Plum.** 1993. Polymerase chain reaction in the diagnosis of *Toxoplasma* encephalitis. *AIDS*. **7**: 1539-1541.
- 114) **Holliman, R. E., J. D. Johnson and D. Saava.** 1990. Diagnosis of cerebral toxoplasmosis in association with AIDS using the polymerase chain reaction. *Scand. J. Infect. Dis.* **22**: 243-244.
- 115) **Bretagne, S., J. M. Costa, M. Vidaud, J. T. Van Nhieu and J. Fleury-Feith.** 1993. Detection of *Toxoplasma gondii* by competitive DNA amplification of bronchoalveolar lavage samples. *J. Infect. Dis.* **168**: 1585-1588.
- 116) **Roth, A., B. Roth, G. Hoffken, S. Steuber, K. I. Khelifa and K. Janitschke.** 1992. Application of the polymerase chain reaction in the diagnosis of pulmonary

- toxoplasmosis in immunocompromised patients. *Eur. J. Clin. Microbiol. Infect. Dis.* **11**: 1177-1181.
- 117) Dupouy-Camet, J., S. L. de Souza, C. Maslo, A. Paugam, A. G. Saimot, R. Benarous, C. Tourte-Shaefer and F. Derouin. 1993. Detection of *Toxoplasma gondii* in venous blood from AIDS patients by polymerase chain reaction. *J. Clin. Microbiol.* **31**: 1866-1869.
 - 118) Filice, G. A., J. A. Hitt, C. D. Mitchell, M. Blackstad and S. W. Sorenson. 1993. Diagnosis of *Toxoplasma* parasitemia in patients with AIDS by gene detection after amplification with polymerase chain reaction. *J. Clin. Microbiol.* **31**: 2327-2331.
 - 119) Ho-Yen, D. O., A. W. L. Joss, A. H. Balfour, E. T. M. Smyth, D. Baird and J. M. W. Chatterton. 1992. Use of the polymerase chain reaction to detect *Toxoplasma gondii* in human blood samples. *J. Clin. Pathol.* **45**: 910-913.
 - 120) Xia, A.-D., Y.-Z. Gu, S.-J. Xu, K.-M. Wang, H.-Z. yang, K.-J. Xu, Z.-L. Qian and S.-S. Chen. 1992. Construction of a genomic DNA library of the *Toxoplasma gondii* ZS₂ strain, screening of specific clones, and DNA diagnosis of toxoplasmosis. *Am. J. Trop. Med. Hyg.* **46**: 50-56.
 - 121) Cazenave, J., A. Cheyrou, P. Blouin, A. M. Johnson and J. Begueret. 1991. Use of polymerase chain reaction to detect *Toxoplasma*. *J. Clin. Pathol.* **44**: 1037.
 - 122) Grover, C. M., P. thulliez, J. S. Remington and J. C. Boothroyd. 1990. Rapid prenatal diagnosis of congenital *Toxoplasma* infection by using polymerase chain reaction and amniotic fluid. *J. Clin. Microbiol.* **28**: 2297-2301.
 - 123) Aouizerate, F., J. Cazenave, L. Poirier, P. H. Verin, A. Cheyrou, J. Begueret and F. Lagoutte. 1993. Detection of *Toxoplasma gondii* in aqueous humor by polymerase chain reaction. *Br. J. Ophthalmol.* **77**: 107-109.
 - 124) Brezin, A. P., C. E. Egwuagu, M. Burnier, Jr., C. Silveira, R. M. Mahdi, R. T. Gazzinelli, R. Belfort, Jr. and R. B. Nussenblatt. 1990. Identification of *Toxoplasma gondii* in paraffin-embedded sections by the polymerase chain reaction. *Am. J. Ophthalmol.* **110**: 599-604.
 - 125) Weiss, L. M., Y.-Y. Chen, G. J. Berry, J. G. Strickler, R. F. Dorfman and R. A. Warnke. 1992. Infrequent detection of *Toxoplasma gondii* genome in toxoplasmic lymphadenitis: a polymerase chain reaction study. *Hum. Pathol.* **23**: 154-158.
 - 126) Holliman, R., J. Johnson, D. Saava, N. Cary and T. Wreghitt. 1992. Diagnosis of *Toxoplasma* infection in cardiac transplant recipients using the polymerase chain reaction. *J. Clin. Pathol.* **45**: 243-244.
 - 127) Burg, J. L., C. M. Grover, P. Pouletty and J. C. Boothroyd. 1989. Direct and sensitive detection of a pathogenic protozoan, *Toxoplasma gondii*, by polymerase chain reaction. *J. Clin. Microbiol.* **27**: 1787-1792.

- 128) **Saava, D., J. C. Morris, J. D. Johnson and R. E. Holliman.** 1990. Polymerase chain reaction for the detection of *Toxoplasma gondii*. *J. Med. Microbiol.* **32**: 1350-1354.
- 129) **Weiss, L. M., S. A. Udem, M. Salgo, H. B. Tanowitz and M. Wittner.** 1991. Sensitive and specific detection of *Toxoplasma* DNA in an experimental murine model: use of *Toxoplasma gondii*-specific cDNA and the polymerase chain reaction. *J. Infect. Dis.* **163**: 180-186.
- 130) **Guay, J.-M., D. Dubois, M.-J. Morency, S. Gagnon, J. Mercier and R. C. Levesque.** 1993. Detection of the pathogenic parasite *Toxoplasma gondii* by specific amplification of ribosomal sequences using complex polymerase chain reaction. *J. Clin. Microbiol.* **31**: 203-207.
- 131) **MacPherson, J. M. and A. A. Gajadhar.** 1993. Sensitive and specific polymerase chain reaction detection of *Toxoplasma gondii* for veterinary and medical diagnosis. *Can. J. Vet. Res.* **57**: 45-48.
- 132) **Blanco, J. C., S. O. Angel, E. Maero, V. Pzsenny, P. Serpente and J. C. Garberi.** 1992. Cloning of repetitive DNA sequences from *Toxoplasma gondii* and their usefulness for parasite detection. *Am. J. Trop. Med. Hyg.* **46**: 350-357.
- 133) **Brindley, P. J., R. T. Gazzinelli, E. Y. Denkers, S. W. Davis, J. P. Dubey, P. Belfort, Jr., M.-C. Martins, C. Silveira, L. Jamra, A. P. Waters and A. Sher.** 1993. Differentiation of *Toxoplasma gondii* from closely related coccidian by riboprint analysis and a surface antigen gene polymerase chain reaction. *Am. J. Trop. Med. Hyg.* **48**: 447-456.
- 134) **Bendall, R. P., P. L. Chiodini.** 1993. New diagnostic methods for parasitic infections. *Current Opinion in Infect. Dis.* **6**: 318-322.
- 135) **Higashi, G. I.** 1984. Immunodiagnostic tests for protozoan and helminthic parasites. *Diagnostic Immunol.* **2**: 1-18.
- 136) **Nantulya, V. M.** 1991. Molecular diagnosis of parasites. *Experientia.* **47(2)**: 142-145.
- 137) **Knowles, D. P. Jr. and J. R. Gorham.** 1993. Advances in the diagnosis of some parasitic diseases by monoclonal antibody-based enzyme-linked immunosorbent assays. *Rev. Sci. Tech.* **12(2)**: 425-433.
- 138) **Yolken, R. H.** 1982. Enzyme immunoassays for the detection of infectious antigens in body fluids: current limitations and future prospects. *Rev. Infect. Dis.* **4(1)**: 35-68.
- 139) **Petri, W. A., Jr.** 1996. Recent advances in amebiasis. *Crit. Rev. Clin. Lab. Sci.* **33(1)**: 1-37.
- 140) **Arguello-Garcia, R., M. C. Sanchez-Guillen, G. Garduno, A. Valadez-Salazar, M. C. Martinez-Garcia, O. Munoz, M. G. Ortega-Pierres.** 1990. Evaluation of an immunoblot methodology for the detection of relevant

Entamoeba histolytica antigens by antibodies induced in human amebiasis. Arch. Invest. Med. (Mex). **21**(Suppl. 1): 3-9.

- 141) Haque, R., L. M. Neville, S. Wood and W. A. Petri Jr. 1994. Short report: detection of *Entamoeba histolytica* and *Entamoeba dispar* directly in stool. Am. J. Trop. Med. Hyg. **50**(5): 595-596.
- 142) Karki, B. M. and S. C. Parija. 1999. Co-agglutination test for the detection of circulating antigens in amebic liver abscess. Am. J. Trop. Med. Hyg. **60**(3): 498-501.
- 143) Haque, R., I. K. Ali, S. Akther and W. A. Petri, Jr. 1998. Comparison of PCR, isoenzyme analysis and antigen detection for diagnosis of *Entamoeba histolytica* infection. J. Clin. Microbiol. **36**(2): 449-452.
- 144) Patel, S., J. McLauchlin and D. P. Casemore. 1997. A simple SDS-PAGE immunoblotting technique using an enhanced chemiluminescence detection system to identify polyclonal antibody responses to complex cryptosporidial antigen preparation following a monoclonal antibody retest and image overlay technique. J. Immunol. Meth. **205**(2): 157-161.
- 145) Majewska, A. C., A. Werner, P. Sulima and T. Luty. 1999. Survey on equine cryptosporidiosis in Poland and the possibility of zoonotic transmission. Ann. Agric. Environ. Med. **6**(2): 161-165.
- 146) Ares-Mazas, M. E., B. Fernandez-da Ponte, C. A. Vergara-Castiblanco, F. Freire-Santos, J. Quilez-Cinca, A. C. Causape-Valenzuela and C. Sanchez-Acedo. 1999. Oocysts, IgG levels and immunoblot patterns determined for *Cryptosporidium parvum* in bovine examined during a visit to a farm (northeastern Spain). Vet. Parasitol. **81**(3): 185-193.
- 147) Ignatius, R., M. Eisenblatter, T. Regnath, U. Mansmann, U. Futh, H. Hahn and J. Wagner. 1997. Efficacy of different methods for detection of low *Cryptosporidium parvum* oocyst numbers or antigen concentrations in stool specimens. Eur. J. Clin. Microbiol. Infect. Dis. **16**(10): 732-736.
- 148) Farrington, M., S. Winters, C. Walker, R. Miller and D. Rubenstein. 1994. *Cryptosporidium* antigen detection in human feces by reverse passive hemagglutination assay. J. Clin. Microbiol. **32**(11): 2755-2759.
- 149) Laxer, M. A., M. E. D'Nicola and R. J. Patel. 1992. Detection of *Cryptosporidium parvum* DNA in fixed, paraffin-embedded tissue by the polymerase chain reaction. Am. J. Trop. Med. Hyg. **47**: 450-455.
- 150) Coker-Vann, M., P. Brown and D. C. Gajdusek. 1984. Serodiagnosis of human cysticercosis using a chromatofocused antigenic preparation of *Taenia solium* cysticerci in an enzyme-linked immunosorbent assay (ELISA). Trans. R. Soc. Trop. Med. Hyg. **78**(4): 492-496.
- 151) Correa, D., E. Sarti, R. Tapia-Romero, R. Rico, I. Alcantara-Anguiano, A. Salgado, L. Valdez and A. Flisser. 1999. Antigens and antibodies in sera from

- human cases of epilepsy or taeniasis from an area of Mexico where *Taenia solium* cysticercosis is endemic. *Ann. Trop. Med. Parasitol.* **93**(1): 69-74.
- 152) Lightowlers, M. W. 1990. Cestode infections in animals: immunological diagnosis and vaccination. *Rev. Sci. Tech.* **9**(2): 463-487.
- 153) Garcia, H. H., L. J. Harrison, R. M. Parkhouse, T. Montenegro, S. M. Martinez, V. C. Tsang and R. H. Gilman. 1998. A specific antigen-detection ELISA for the diagnosis of human neurocysticercosis. The Cysticercosis Working Group in Peru. *Trans. R. Soc. Trop. Med. Hyg.* **92**(4): 411-414.
- 154) Sciutto, E., J. J. Martinez, N. M. Villalobos, M. Hernandez, M. V. Jose, C. Beltran, F. Rodarte, I. Flores, J. R. Bobadilla, G. Fragoso, M. E. Parkhouse, L. J. Harrison and A. S. de Aluja. 1998. Limitations of current diagnostic procedures for the diagnosis of *Taenia solium* cysticercosis in rural pigs. *Vet. Parasitol.* **79**(4): 299-313.
- 155) Onyango-Abuje, J. A., G. Hughes, M. Opicha, K. M. Nginyi, M. K. Rugutt, S. H. Wright and L. J. Harrison. 1996. Diagnosis of *Taenia saginata* cysticercosis in Kenyan cattle by antibody and antigen ELISA. *Vet. Parasitol.* **61**(3-4): 221-230.
- 156) Stefaniak, J. 1997. Fine needle aspiration biopsy in the differential diagnosis of the liver cystic echinococcosis. *Acta Trop.* **67**(1-2): 107-111.
- 157) Deplazes, P. and J. Eckbert. 1996. Diagnosis of *Echinococcus multilocularis* infection in final hosts. *Appl. Parasitol.* **37**(4): 245-252.
- 158) Matsuo, K., M. Shimizu, N. Nonaka, Y. Oku and M. Kamiya. 2000. Development and sexual maturation of *Echinococcus vogeli* in an alternative definitive host, Mongolian gerbil (*Meriones unguiculatus*). *Acta Trop.* **75**(3): 323-330.
- 159) Jenkins, D. J., A. Fraser, H. Bradshaw and P. S. Craig. 2000. Detection of *Echinococcus granulosus* coproantigens in Australian canids with natural or experimental infection. *J. Parasitol.* **86**(1): 140-145.
- 160) Morishima, Y., H. Tsukada, N. Nonaka, Y. Oku and M. Kamiya. 1999. Evaluation of coproantigen diagnosis for natural *Echinococcus multilocularis* infection in red foxes. *Jpn. J. Vet. Res.* **46**(4): 185-189.
- 161) Nonaka, N., H. Tsukada, N. Abe, Y. Oku and M. Kamiya. 1999. Monitoring of *Echinococcus multilocularis* infection in red foxes in Shiretoko, Japan, by coproantigen detection. *Parasitology*. **117**(Pt2): 193-200.
- 162) Kanwar, J. R., R. K. Kanwar, A. S. Grewal and V. K. Vinayak. 1994. Significance of detection of immune-complexed 8 kDa hydatid-specific antigen for immunodiagnosis of hydatidosis. *FEMS Immunol. Med. Microbiol.* **9**(3): 231-236.

- 163) Poretti, D., E. Felleisen, F. Grimm, M. Pfister, F. Teuscher, C. Zuercher, J. Reichen and B. Gottstein. 1999. Differential immunodiagnosis between cystic hydatid disease and other cross-reactive pathologies. *Am. J. Trop. Med. Hyg.* **60**(2): 193-198.
- 164) Dumenigo, B. E., A. M. Espino, C. M. Finlay and M. Mezo. 2000. Kinetics of antibody-based antigen detection in serum and faeces of sheep experimentally infected with *Fasciola hepatica*. *Vet. Parasitol.* **89**(1-2): 153-161.
- 165) Cordova, M., L. Reategui and J. R. Espinoza. 1999. Immunodiagnosis of human fascioliasis with *Fasciola hepatica* cysteine proteinases. *Trans. R. Soc. Trop. Med. Hyg.* **93**(1): 54-57.
- 166) Pelayo, L., A. M. Espino, B. E. Dumenigo, C. M. Finlay. 1998. The detection of antibodies, antigens and circulating immune complexes in acute and chronic fascioliasis. Preliminary Results. *Rev. Cubana. Med. Trop.* **50**(3): 209-214.
- 167) Espino, A. M., A. Diaz, A. Perez and C. M. Finlay. 1998. Dynamics of antigenemia and coproantigens during a human *Fasciola hepatica* outbreak. *J. Clin. Microbiol.* **36**(9): 2723-2726.
- 168) Abdel-Rahman, S. M., K. L. O'Reilly and J. B. Malone. 1998. Evaluation of a diagnostic monoclonal antibody-based capture enzyme-linked immunosorbent assay for the detection of a 26- to 28-kd *Fasciola hepatica* coproantigen in cattle. *Am. J. Vet. Res.* **59**(5): 533-537.
- 169) Espino, A. M. and C. M. Finlay. 1994. Sandwich enzyme-linked immunosorbent assay for detection of excretory secretory antigens in human fascioliasis. *J. Clin. Microbiol.* **32**(1): 190-193.
- 170) Youssef, F. G., N. S. Mansour and A. G. Aziz. 1991. Early diagnosis of human fascioliasis by the detection of copro-antigens using counterimmunoelectrophoresis. *Trans. R. Soc. Trop. Med. Hyg.* **85**(3): 383-384.
- 171) Shaker, Z. A., Z. A. Demerdash, W. A. Mansour, H. I. Hassanein, H. G. el Baz and H. I. Gindy. 1994. Evaluation of specific *Fasciola* antigen in the immunodiagnosis of human fascioliasis in Egypt. *J. Egypt. Soc. Parasitol.* **24**(3): 463-470.
- 172) Shaheen, H. I., K. A. Kamal, Z. Farid, N. Mansour, F. N. Boctor and J. N. Woody. 1989. Dot-enzyme-linked immunosorbent assay (ELISA) for the rapid diagnosis of human fascioliasis. *J. Parasitol.* **75**(4): 549-552.
- 173) More, S. J. and D. B. Copeman. 1990. A highly specific and sensitive monoclonal antibody-based ELISA for the detection of circulating antigen in bancroftian filariasis. *Trop. Med. Parasitol.* **41**: 403-406.
- 174) Partono, F., S. Oernijati and I. Hudojo. 1977. Malayan filariasis in Central Sulawesi (Celebes), Indonesia. *Southeast Asian J. Trop. Med. Pub. Health.* **8**: 452-458.

- 175) **Turner, P., B. Copeman, D. Gerisi and R. Speare.** 1993. A comparison of the Og4C3 antigen capture ELISA, the Knott test and the IgG₄ assay and clinical signs, in the diagnosis of Bancroftian filariasis. *Trop. Med. Parasitol.* **44(1)**: 45-48.
- 176) **Lalitha, P., M. Ravichandran, S. Suba, P. Kalilaj, R. B. Narayanan and K. Jayaraman.** 1998. Quantitative assessment of circulating antigens in human lymphatic filariasis: a field evaluation of monoclonal antibody-based ELISA using blood collected on filter strips. *Trop. Med. Int. Health.* **3**: 41-45.
- 177) **Lammie, P. J., M. D. Reiss, K. A. Dimock, T. G. Streit, J. M. Roberts and M. L. Eberhard.** 1998. Longitudinal analysis of the development of filarial infection and antifilarial immunity in a cohort of Haitian children. *Am. J. Trop. Med. Hyg.* **59**: 217-221.
- 178) **Rocha, A. D. Addiss, M. E. Ribeiro, J. Noroes, Z. Medeiros and G. Deryer.** 1996. Evaluation of the Og4c3 ELISA in *Wuchereria bancrofti* infection: infected persons with undetectable or ultra-low microfilarial densities. *Trop. Med. Int. Healt.* **1**: 859-864.
- 179) **Simonsen, P. E. and S. K. Dunyo.** 1999. Comparative evaluation of three new tools for diagnosis of Bancroftian filariasis based on the detection of circulating antigens. *Trans. R. Soc. Trop. Med. Hyg.* **93**: 278-282.
- 180) **Weil, G. L., P. J. Lammie and N. Weiss.** 1997. The ICT Filariasis Test: A rapid-format antigen tests for diagnosis of Bancroftian filariasis. *Parasitol. Today.* **13(10)**: 401-404.
- 181) **Weil, G. L. and F. Liftis.** 1987. Identification and partial characterization of a parasite antigen in sera from humans infected with *Wuchereria bancrofti*. *J. Immunol.* **138(9)**: 3035-3041.
- 182) **Harnett, W., J. E. Bradley and T. Garate.** 1998. Molecular and immunodiagnosis of human filarial nematode infections. *Parasitol.* **117(Suppl)**: S59-S71.
- 183) **McReynolds, L. A., C. Poole, Y. Hong, S. A. Williams, F. Partono and J. Bradley.** 1993. Recent advances in the application of molecular biology in filariasis. *Southeast Asian J. Trop. Med. Pub. Health.* **24(Suppl2)**: 55-63.
- 184) **Lim, P. K.** 1993. Recent advances in diagnostic techniques in filariasis. *Southeast Asian J. Trop. Med. Pub. Health.* **24(Suppl2)**: 45-50.
- 185) **Kobayashi, M., M. Niimura, t. Kanazawa, M. K. Husky, E. Malagueno and J. V. Santana.** 1997. Detection of microfilarial antigen in circulating immune complex from sera of *Wuchereria bancrofti*-infected individuals. *Am. J. Trop. Med. Hyg.* **57(2)**: 200-204.
- 186) **Theodore, J. G. and P. Kaliraj.** 1996. *Wuchereria bancrofti* recombinant antigen-derived poly- and monoclonal antibodies for the detection of circulating antigen(s) in the sera of lymphatic filarial patients. *J. Helminthol.* **70(1)**: 69-74.

- 187) Weil. G. L., D. C. Jain, S. Santhanam, A. Malhotra, H. Kumar, K. V. P. Sethumadhavan, F. Lifitis and T. K. Gosh. 1987. A monoclonal antibody-based enzyme linked immunoassay for detecting parasite antigenemia in bancroftian filariasis. *J. Infect. Dis.* **156**(2): 350-355.
- 188) Butcher, P. D. and M. J. G. Farthing. 1989. DNA probes for the faecal diagnosis of *Giardia lamblia* infections in man. *Biochem. Soc. Trans.* **17**: 363-364.
- 189) Mahbubani, M. H., A. K. Bej, M. Perlin, F. W. Shaeffer III, W. Jakubowski and R. M. Atlas. 1991. Detection of *Giardia lamblia* cysts by using the polymerase chain reaction and distinguishing live from dead cysts. *Appl. Environ. Microbiol.* **57**: 3456-3461.
- 190) Mahbubani, M. H., A. K. Bej, M. Perlin, F. W. Shaeffer III, W. Jakubowski and R. M. Atlas. 1992. Differentiation of *Giardia duodenalis* from other *Giardia spp.* by using polymerase chain reaction and gene probes. *J. Clin. Microbiol.* **30**: 74-78.
- 191) Weiss, J. B., H. van Keulen and T. E. Nash. 1992. Classification of subgroups of *Giardia lamblia* based upon ribosomal RNA gene sequence using polymerase chain reaction. *Mol. Biochem. Parasitol.* **54**: 73-86.
- 192) Faubert, G. 2000. Immune response to *Giardia duodenalis*. *Clin. Microbiol. Rev.* **13**(1): 35-54.
- 193) Hill, D. R. 1993. Giardiasis. Issues in diagnosis and management. *Infect. Dis. Clin. North. Am.* **7**(3): 503-525.
- 194) Pickering, L. K. and P. G. Engelkirk. 1988. *Giardia lamblia*. *Pediatr. Clin. North. Am.* **35**(3): 565-577.
- 195) Hanson, K. L. and C. P. Cartwright. 2001. Use of an enzyme immunoassay does not eliminate the need to analyze multiple stool specimens for sensitive detection of *Giardia lamblia*. *J. Clin. Microbiol.* **39**(2): 474-477.
- 196) Torres, D., M. Fernandez, T. Brito and C. Finlay. 1997. Solid-phase immunoenzyme assay for detecting *Giardia lamblia* antigens. *Rev. Cubana. Med. Trop.* **49**(1): 52-58.
- 197) Jelinek, T., G. Peyerl, T. Loscher and H. D. Nothdurft. 1996. Giardiasis in travelers: evaluation of an antigen-capture ELISA for the detection of *Giardia lamblia*-antigen in stool. *Z. Gastroenterol.* **34**(4): 237-240.
- 198) Bitkowska, E., M. Walochowa and T. H. Dzbenski. 1995. Detection of *Giardia* antigens in feces by immunoenzymatic method; advantages and disadvantages of commercially available diagnostic kits. *Med. Dosw. Mikrobiol.* **47**(1-2): 95-99.
- 199) Vinayak, V. K., P. Dutt and S. Mehta. 1993. Uses and limitations of monoclonal antibodies to *Giardia lamblia*-specific 66-kDa copro-antigen in copro-immunodiagnosis of giardiasis. *FEMS Immunol. Med. Microbiol.* **6**(1): 37-44.

- 200) Chappell, C. L. and C. C. Matson. 1992. *Giardia* antigen detection in patients with chronic gastrointestinal disturbances. *J. Fam. Pract.* **35**(1): 49-53.
- 201) Knisley, C. V., P. G. Engelkirk, L. K. Pickering, M. S. West and E. N. Janoff. 1989. Rapid detection of *Giardia* antigen in stool with the use of enzyme immunoassays. *Am. J. Clin. Pathol.* **91**(6): 704-708.
- 202) Das Gupta, S., D. K. Ghosh and H. K. Majumder. 1991. A cloned kinetoplast DNA minicircle fragment from a *Leishmania* spp. specific for post-kala-azar dermal leishmaniasis strains. *Parasitol.* **102**: 187-191.
- 203) Gramiccia, M., D. F. Smith, M. C. Angelici, P. D. Ready and L. Gradoni. 1992. A kinetoplast DNA probe diagnostic for *Leishmania infantum*. *Parasitol.* **105**: 29-34.
- 204) Guevara, P., G. Alonso, J. F. da Silveira, M. de Mello, J. V. Scorza, N. Anez and J. L. Ramirez. 1992. Identification of new world *Leishmania* using ribosomal gene spacer probes. *Mol. Biochem. Parasitol.* **56**: 15-26.
- 205) Howard, M. K., J. M. Kelly, R. P. Lane and M. A. Miles. 1991. A sensitive repetitive DNA probe that is specific to the *Leishmania donovani* complex and its use as an epidemiological and diagnostic reagent. *Mol. Biochem. Parasitol.* **44**: 63-72.
- 206) Lopez, M., R. Inga, M. Cangalaya, J. Echevarria, A. Llanos-Cuentas, C. Orrego and J. Arevalo. 1993. Diagnosis of *Leishmania* using the polymerase chain reaction: a simplified procedure for field work. *Am. J. Trop. Med. Hyg.* **49**: 348-356.
- 207) Smith, D. F., S. Searle, P. D. Ready, M. Gramiccia and R. Ben-Ismael. 1989. A kinetoplast DNA probe diagnostic for *Leishmania major*: sequence homologies between regions of *Leishmania* minicircles. *Mol. Biochem. Parasitol.* **37**: 213-224.
- 208) Smyth, A. J., A. Ghosh, M. Q. Hassan, D. Basu, M. H. L. De Bruijn, S. Adhya, K. K. Mallick and D. C. Barker. 1992. Rapide and sensitive detection of *Leishmania* kinetoplast DNA from spleen and blood samples of kala-azar patients. *Parasitol.* **105**: 183-192.
- 209) Kar, K. 1995. Serodiagnosis of leishmaniasis. *Crit. Rev. Microbiol.* **21**(2): 123-152.
- 210) Santos-Gomes, G., S. Gomes-Pereira, L. Campino, M. D. Araujo and P. Abranches. 2000. Performance of immunoblotting in human immunodeficiency virus-*Leishmania* sp.-coinfected patients. *J. Clin. Microbiol.* **38**(1): 175-178.
- 211) Ismail, A., A. Kharazmi, H. Permin and A. M. el Hassan. 1997. Detection and characterization of *Leishmania* in tissues of patients with post kala-azar dermal leishmaniasis using a specific monoclonal antibody. *Trans. R. Soc. Trop. Med. Hyg.* **91**(3): 283-285.

- 212) Marty, P., A. Lelievre, J. F. Quaranta, A. Rahal, M. Gari-Toussaint and Y. Le Fichoux. 1994. Use of the leishmanin skin test and western blot analysis for epidemiological studies in visceral leishmaniasis areas: experience in a highly endemic focus in Alpes-Maritimes (France). *Trans. R. Soc. Trop. Med. Hyg.* **88**(6): 658-659.
- 213) Sundar, S., S. G. Reed, V. P. Singh, P. C. Kumar and H. W. Murray. 1997. Rapid accurate diagnosis of Indian visceral leishmaniasis. *Lancet.* **351**(9102): 563-565.
- 214) Mengistu, G., H. Akuffo and T. E. Fehniger. 1992. Immunoblot analysis of sera from Ethiopian cutaneous leishmaniasis by antibody class. *Scand. J. Immunol.* **11**(Suppl): 149-152.
- 215) Hanscheid, T. 1999. Diagnosis of malaria: a review of alternatives to conventional microscopy. *Clin. Lab. Haematol.* **21**(4): 235-245.
- 216) Makler, M. T., C. J. Palmer and A. L. Ager. 1998. A review of practical techniques for the diagnosis of malaria. *Ann. Trop. Med. Parasitol.* **92**(4): 419-433.
- 217) Graves, P. M., R. Boreham, G. Robert, L. Fray, L. J. Xu, Y. M. Huang, W. Relf, A. Saul and C. Kidson. 1992. Antibody detection ELISAs for malaria diagnosis. *Southeast. Asian. J. Trop. Med. Pub. Health.* **23**(4): 752-761.
- 218) Stephens, J. K., K. Phanart, W. Rooney and G. Barnish. 1999. A comparison of three malaria diagnostic tests, under field conditions in North-west Thailand. *Southeast. Asian. J. Trop. Med. Pub. Health.* **30**(4): 625-630.
- 219) Taylor, H. M., S. A. Kyes, D. Harris, N. Kriek and C. I. Newbold. 2000. A study of var gene transcription *in vitro* using universal var gene primers. *Mol. Biochem. Parasitol.* **105**(1): 13-23.
- 220) Iqbal, J., A. Sher, P. R. Hira and R. Al-Owaish. 1999. Comparison of the OptiMAL test with PCR diagnosis of malaria in immigrants. *J. Clin. Microbiol.* **37**(11): 3644-3646.
- 221) Cooke, A. H., P. L. Chiodini, T. Doherty, A. H. Moody and M. Pinder. 1999. Comparison of a parasite lactate dehydrogenase-based immunochromatographic antigen detection assay (OptiMAL) with microscopy for the detection of malaria parasites in human blood samples. *Am. J. Trop. Med. Hyg.* **60**(2): 173-176.
- 222) Rodriguez-Acosta, A., N. G. Dominguez, I. Aguilar and M. E. Giron. 1998. Characterization of *Plasmodium falciparum* glutamate dehydrogenase-soluble antigen. *Braz. J. Med. Biol. Res.* **31**(9): 1149-1155.
- 223) Van den Ende, J., T. Vervoort, A. Van Gompel and L. Lynen. 1998. Evaluation of two tests based on the detection of histidine rich protein 2 for the diagnosis of imported *Plasmodium falciparum* malaria. *Trans. R. Soc. Trop. Med. Hyg.* **92**(3): 285-288.

- 224) Bellagra, N., F. Ajana and M. Caillaux. 1998. ParaSight F in the diagnosis of *Plasmodium falciparum* malaria. *Pathol. Biol. (Paris)*. **46(5)**: 301-306.
- 225) Desakorn, V., K. Silamut, B. Angus, D. Sahassananda, K. Chotivanich, P. Suntharasamai, J. Simpson and N. J. White. 1997. Semi-quantative measurement of *Plasmodium falciparum* antigen PfHRP2 in blood and plasma. *Trans. R. Soc. Trop. Med. Hyg.* **91(4)**: 479-483.
- 226) Di Perri, G., P. Oliaro, S. Nardi, B. Allegranzi, R. Deganello, S. Vento, M. Lanzafame, A. Cazzadori, S. Bonora and E. Concia. 1997. The ParaSight-F rapid dipstick antigen capture assay for monitoring parasite clearance after drug treatment of *Plasmodium falciparum* malaria. *Trans. R. Soc. Trop. Med. Hyg.* **91(4)**: 403-405.
- 227) Mharakurwa, S. and C. J. Shiff. 1997. Post treatment sensitivity studies with the ParaSight-F test for malaria diagnosis in Zimbabwe. *Acta Trop.* **66(2)**: 61-67.
- 228) Humar, A., C. Ohrt, M. A. Harrington, D. Pillai and K. C. Kain. 1997. Parasight F test compared with the polymerase chain reaction and microscopy for the diagnosis of *Plasmodium falciparum* in travelers. *Am. J. Trop. Med. Hyg.* **56(1)**: 44-48.
- 229) Zhan, B., L. Zhang, J. Wang and X. Feng. 1994. Detection of *Plasmodium falciparum* by polymerase chain reaction (PCR). *Chong. Bing. Za. Zhi.* **12(2)**: 111-114.
- 230) Barker, R. H., Jr., T. Banchongaksorn, J. M. Courval, W. Suwonkerd, K. Rimwongtragoon and D. F. Wirth. 1992. A simple method to detect *Plasmodium falciparum* directly from blood samples using the polymerase chain reaction. *Am. J. Trop. Med. Hyg.* **46**: 416-426.
- 231) Brown, A. E., K. C. Kain, J. Pipithkul and H. K. Webster. 1992. Demonstration by the polymerase chain reaction of mixed *Plasmodium falciparum* and *P. vivax* infections undetected by conventional microscopy. *Trans. R. Soc. Trop. Med. Hyg.* **86**: 609-612.
- 232) Seesod, N., J. Lundeberg, A. Hedrum, L. Aslund, A. Holder, S. Thaithong and M. Uhlen. 1993. Immunomagnetic purification to facilitate DNA diagnosis of *Plasmodium falciparum*. *J. Clin. Microbiol.* **31**: 2715-2719.
- 233) Snounou, G., S. Viriyakosol, W. Jarra, S. Thaithong and K. N. Brown. 1993. Identification of the four human malarial parasite species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infections. *Mol. Biochem. Parasitol.* **58**: 283-292.
- 234) Snounou, G., S. Viriyakosol, X. P. Zhu, W. Jarra, L. Pinheiro, V. E. do Rosario, S. Thaithong and K. N. Brown. 1993. High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. *Mol. Biochem. Parasitol.* **61**: 315-320.

- 235) **Wataya, Y. M. Arai, F. Kubochi, C. Mizukoshi, T. Kakutani, N. Ohta and A. Ishii.** 1993. DNA diagnosis of falciparum malaria using a double PCR technique: a field trial in the Solomon Islands. *Mol. Biochem. Parasitol.* **58**: 165-168.
- 236) **Parkhouse, R. M., Z. Cabrera and W. Harnett.** 1987. *Onchocerca* antigens in protection, diagnosis and pathology. *Vet. Immunol. Immunopathol.* **17**(1-4): 313-324.
- 237) **Vincent, J. A., S. Lustigman, S. Zhang and G. J. Weil.** 2000. A comparison of newer tests for the diagnosis of onchocerciasis. *Ann. Trop. Med. Parasitol.* **94**(3): 253-258.
- 238) **Chandrashekar, R., A. F. Ogunrinade, R. W. Henry, S. Lustigman and G. J. Weil.** 1993. *Onchocerca volvulus*: monoclonal antibodies to immune complex-associated parasite antigens. *Exp. Parasitol.* **77**(2): 224-234.
- 239) **Bradley, J. E., K. R. Trenholme, A. J. Gillespie, R. Guderian, V. Titanji, Y. Hong and L. McReynolds.** 1993. A sensitive serodiagnostic test for onchocerciasis using a cocktail of recombinant antigens. *Am. J. Trop. Med. Hyg.* **48**(2): 198-204.
- 240) **Chandrashekar, R., A. F. Ogunrinade, R. M. Alvarez, O. O. Kale and G. J. Weil.** 1990. Circulating immune complex-associated parasite antigens in human onchocerciasis. *J. Infect. Dis.* **162**(5): 1159-1164.
- 241) **Erttmann, K. D., S. E. O. Meredith, B. M. Greene and T. R. Unnasch.** 1990. Isolation and characterization of form specific DNA sequences of *Onchocerca volvulus*. *Acta Leiden.* **59**: 253-260.
- 242) **Harnett, W., A. E. Chambers, A. Renz and R. M. E. Parkhouse.** 1989. An oligonucleotide probe specific for *Onchocerca volvulus*. *Mol. Biochem. Parasitol.* **35**: 119-126.
- 243) **Meredith, S. E. O., G. Lando, A. A. Gbakima, P. A. Zimmerman and T. R. Unnasch.** 1991. *Onchocerca volvulus*: application of the polymerase chain reaction to identification and strain differentiation of the parasite. *Exp. Parasitol.* **73**: 335-344.
- 244) **Shah, J. S., M. Karam, W. F. Piessens and D. F. Wirth.** 1987. Characterization of an *Onchocerca*-specific DNA clone from *Onchocerca volvulus*. *Am. J. Trop. Med. Hyg.* **37**: 376-384.
- 245) **Jyding Vennervald, B., A. I. Kahama and C. M. Reimert.** 2000. Assessment of morbidity in *Schistosoma haematobium* and future tools. *Acta Trop.* **77**(1): 81-89.
- 246) **Royers, R., W. Jacobs, J. J. Bogers, A. M. Deelder and E. Van Marck.** 2000. Study of the distribution patterns of *Schistosoma haematobium* egg antigens recognized by six different monoclonal antibodies in the parasite and the host. *Parasite.* **7**(4): 297-303.

- 247) Tarp, B., F. T. Black and E. Petersen. 2000. The immunofluorescence antibody test (IFAT) for the diagnosis of schistosomiasis used in a non-endemic area. *Trop. Med. Int. Health.* **5**(3): 185-191.
- 248) Salah, F., Z. Demerdash, Z. Shaker, A. El Bassiouny, G. El Attar, S. Ismail, N. Badir, A. S. El Din and M. Mansour. 2000. A monoclonal antibody against *Schistosoma haematobium* soluble egg antigen: efficacy for diagnosis and monitoring of cure of *Schistosoma haematobium* infection. *Parasitol. Res.* **86**(1): 74-80.
- 249) Atallah, A. M., E. Yones, H. Ismail, S. A. Masry, A. Tabll, A. A. Elenein and N. A. Ghawalby. 1999. Immunochemical characterization and diagnostic potential of a 63-kilodalton *Schistosoma* antigen. *Am. J. Trop. Med. Hyg.* **60**(3): 493-497.
- 250) Al-Sherbiny, M. M., A. M. Osman, K. Hancock, A. M. Deelder and V. C. Tsang. 1999. Application of immunodiagnostic assays: detection of antibodies and circulating antigens in human schistosomiasis and correlation with clinical findings. *Am. J. Trop. Med. Hyg.* **60**(6): 960-966.
- 251) Shaker, Z. A., M. A. Kaddah, S. B. Hanallah and M. I. El-Khodary. 1998. Production of monoclonal antibodies against target schistosomal antigen secreted in the urine of *Schistosoma mansoni*-infected patients. *Int. J. Parasitol.* **28**(12): 1893-1901.
- 252) Attallah, A. M., H. Ismail, S. A. el Masry, H. Rizk, A. E. Handousa, M. el Bendary, A. Tabll and F. Ezzat. 1999. Rapid detection of a *Schistosoma mansoni* circulating antigen excreted in urine of infected individuals by using a monoclonal antibody. *J. Clin. Microbiol.* **37**(2): 354-357.
- 253) Attallah, A. M., S. A. el Masry, H. Rizk, H. Ismail, M. el Bendary, A. E. Handousa, A. M. el Shazly and M. A. Arafat. 1997. Fast-Dot ELISA using urine, a rapid and dependable field assay for diagnosis of schistosomiasis. *J. Egypt. Soc. Parasitol.* **27**(1): 279-289.
- 254) Rabello, A. 1997. Diagnosing schistosomiasis. *Mem. Inst. Oswaldo. Cruz.* **92**(5): 669-676.
- 255) Peters, P. A., M. El Alamy, K. S. Warren and A. A. F. Mahmoud. 1980. Quick Kato smear for field quantification of *Schistosoma mansoni* eggs. *Am. J. Trop. Med. Hyg.* **29**: 217-219.
- 256) Xue, C. G., M. G. Taylor, Q. D. Bickle, L. Savioli and E. A. Renganathan. 1993. Diagnosis of *Schistosoma haematobium* infection: evaluation of ELISA using keyhole limpet haemocyanin or soluble egg antigen in comparison with detection of eggs or haematuria. *Trans. R. Soc. Trop. Med. Hyg.* **87**(6): 654-658.
- 257) de Jonge, N., P. G. Kremsner, F. W. Krijger, G. Schommer, Y. E. Fillie, D. Kornelis, R. J. van Zeyl, G. J. van Dam, H. Feldmeier and A. M. Deelder. 1990. Detection of the schistosome circulating cathodic antigen by enzyme

immunoassay using biotinylated monoclonal antibodies. Trans. R. Soc. Trop. Med. Hyg. **84**(6): 815-818.

- 258) Van't Wout, A. B., N. De Jonge, W. U. Tiu, E. E. Garcia, G. F. Mitchell and A. M. Deelder. 1992. Schistosome circulating anodic antigen in serum of individuals infected with *Schistosoma japonicum* from the Philippines before and after chemotherapy with praziquantel. Trans. R. Soc. Trop. Med. Hyg. **86**(4): 410-413.
- 259) Van Lieshout, L., N. de Jonge, N. A. el Masry, M. M. Mansour, F. W. Krijger and A. M. Deelder. 1992. Improved diagnostic performance of the circulating antigen assay in human schistosomiasis by parallel testing for circulating anodic and cathodic antigens in serum and urine. Am. J. Trop. Med. Hyg. **47**(4): 463-469.
- 260) Bilbao, A., J. M. Garcia, I. Pocheville, C. Gutierrez, J. M. Corral, A. Samper, G. Rubio, J. Benito, P. Villas, D. Fernandez and J. I. Pijoan. 1999. Round Table: Urticaria in relation to infections. Allergol. Immunopathol. (Madr.). **27**(2): 73-85.
- 261) Hassan, M. M., M. A. Badawi and M. Strand. 1992. Circulating schistosomal antigen in diagnosis and assessment of cure in individuals infected with *Schistosoma mansoni*. J. Trop. Med. Hyg. **46**(6): 737-744.
- 262) van Knapen F. and S. O. Panggabean. 1977. Detection of circulating antigen during acute infections with *Toxoplasma gondii* by enzyme-linked immunosorbent assay. J. Clin Microbiol. **6**(6): 545-547.
- 263) Raizman, R. E. and F. A. Neva. 1975. Detection of circulating antigen in acute experimental infections with *Toxoplasma gondii*. J. Infect. Dis. **132**(1): 44-48.
- 264) Hughes, H. P. A. 1981. Characterization of the circulating antigen of *Toxoplasma gondii*. Immunol. Letters. **3**(1): 99-102.
- 265) Huskinson, J., P. Stepick-Biek and J. S. Remington. 1989. Detection of antigens in urine during acute toxoplasmosis. J. Clin. Microbiol. **27**(5): 1099-1101.
- 266) Fachado, A., L. Fonte, L. Rojas, E. Alberti and R. Machin. 1990. Technique for the detection of *Toxoplasma gondii* antigens in mouse urine. Mem. Inst. Oswaldo Cruz. **85**(1): 65-68.
- 267) Hafid, J., T. M. Sung, H. Raberin, Z. Y. Akono, B. Pozzetto, M. Jana. 1995. Detection of circulating antigens of *Toxoplasma gondii* in human infection. Am. J. Trop. Med. Hyg. **52**(4): 336-339.
- 268) Araujo, F. G., J. S. Remington. 1980. Antigenemia in a recently acquired acute toxoplasmosis. J. Infect. Dis. **141**(2): 144-150.

- 269) Brooks, R. G., S. D. Sharma, J. S. Remington. 1985. Detection of *Toxoplasma gondii* antigens by a dot-immunobinding technique. J. Clin. Microbiol. **21**(1): 113-116.
- 270) Potasman, I., F. G. Araujo, P. Thulliez, G. Desmonts and J. S. Remington. 1987. *Toxoplasma gondii* antigens recognized by sequential samples of serum obtained from congenitally infected infants. J. Clin. Microbiol. **25**(10): 1926-1931.
- 271) Derouin, F., P. Thulliez, E. Candolfi, F. Daffos, F. Forestier. 1988. Early prenatal diagnosis of congenital toxoplasmosis using amniotic fluid samples and tissue culture. Eur. J. Clin. Microbiol. Infect. Dis. **7**(3): 423-425.
- 272) Fachado, A., L. Fonseca, L. Fonte, E. Alberti, R. Cox and F. Bandera. 1997. *Toxoplasma gondii* antigenuria in patients with acquired immune deficiency syndrome. Mem. Inst. Oswaldo Cruz. **92**(5): 589-593.
- 273) Dupouy-Camet, J., S. Lavareda de Souza, C. Maslo, A. Paugam, A. G. Saimot, R. Benarous, C. Tourte-Schaefer and F. Derouin. 1993. Detection of *Toxoplasma gondii* in venous blood from AIDS patients by polymerase chain reaction. J. Clin. Microbiol. **31**(7): 1866-1869.
- 274) Dannemann, B. R., D. M. Israelski, G. S. Leoung, T. McGraw, J. Mills and J. S. Remington. 1991. Toxoplasma serology, parasitemia and antigenemia in patients at risk for toxoplasmic encephalitis. AIDS. **5**(11): 1363-1365.
- 275) Li, S., G. Maine, Y. Suzuki, F. G. Araujo, G. Galvan, J. S. Remington and S. Parmley. 2000. Serodiagnosis of recently acquired *Toxoplasma gondii* infection with a recombinant antigen. J. Clin. Microbiol. **38**(1): 179-184.
- 276) Yopez-Mulia, L., C. Arriaga, N. Viveros, A. Adame, E. Benitez and M. G. Ortega-Pierres. 1999. Detection of *Trichinella* infection in slaughter horses by ELISA and western blot analysis. Vet. Parasitol. **81**(1): 57-68.
- 277) Nockler, K., E. Pozio, W. P. Voigt and J. Heidrich. 2000. Detection of *Trichinella* in food animals. Vet. Parasitol. **93**(3-4): 335-350.
- 278) Makarem, S. S., T. A. Morsy and S. F. el-Seoud. 1989. The use of three immunologic tests in detection of antibodies against *Trichinella spiralis*. J. Egypt. Soc. Parasitol. **19**(1): 331-336.
- 279) Ivanoska, D., K. Cuperlovic, H. R. Gamble and K. D. Murrell. 1989. Comparative efficacy of antigen and antibody detection tests for human trichinellosis. J. Parasitol. **75**(1): 38-41.
- 280) Gomez-Priego, A., L. Crecencio-Rosales, J. L. de-La-Rosa. 2000. Serological evaluation of thin-layer immunoassay-enzyme-linked immunosorbent assay for antibody detection in human trichinellosis. Clin. Diagn. Lab. Immunol. **7**(5): 810-812.

- 281) **Poletaeva, O. G. and N. N. Krasovskaia.** 1995. The efficiency of the serological reactions with *Trichinella spiralis* somatic antigen in the diagnosis of human trichinellosis caused by infection with natural and synanthropic *Trichinella* isolates. *Med. Parazitol (Mosk)*. **2**: 33-35.
- 282) **Ruangkunaporn, Y., G. Watt, C. Karnasuta, K. Jongsakul, P. Mahannop, M. Chongsa-nguan and W. Chaicumpa.** 1994. Immunodiagnosis of trichinellosis: efficacy of somatic antigen in early detection of human trichinellosis. *Asian. Pac. J. Allergy. Immunol.* **12(1)**: 39-42.
- 283) **Dick, T. A., M. Lu, T. deVos and K. Ma.** 1992. The use of the polymerase chain reaction to identify porcine isolates of *Trichinella*. *J. Parasitol.* **78**: 145-148.
- 284) **Dupouy-Camet, J., C. Soule, J.-P. Guillou, E. Rouer, S. L. de Souza, T. Ancelle and R. Benarous.** 1991. Detection of repetitive sequences of *Trichinella spiralis* by the polymerase chain reaction in experimentally infected mice. *Parasitol. Res.* **77**: 180-182.
- 285) **Marcelain, K., A. Colombo, M. C. Molina, L. Ferreira, M. Lorca, J. C. Aguillon and A. Ferreira.** 2000. Development of an immunoenzymatic assay for the detection of human antibodies against *Trypanosoma cruzi* calreticulin, an immunodominant antigen. *Acta Trop.* **75(3)**: 291-300.
- 286) **Asonganyi, T. F. Doua, S. N. Kibona, Y. M. Nyasulu, R. Masake and F. Kuzoe.** 1998. A multi-centre evaluation of the card indirect agglutination test for trypanosomiasis (TrypTect CIATT). *Ann. Trop. Med. Parasitol.* **92(8)**: 837-844.
- 287) **Anez, N., H. Carrasco, H. Parada, G. Crisante, A. Rojas, C. Fuenmayor, N. Gonzalez, G. Percoco, R. Borges, P. Guevara and J. L. Ramirez.** 1999. Myocardial parasite persistence in chronic chagasic patients. *Am. J. Trop. Med. Hyg.* **60(5)**: 726-732.
- 288) **Carriazo, C. S., A. Sembaj, A. M. Aguerri, J. M. Requena, C. Alonso, J. Bua, A. Ruiz, E. Segura and J. M. Barral.** 1998. Polymerase chain reaction procedure to detect *Trypanosoma cruzi* in blood samples from chronic chagasic patients. *Diagn. Microbiol. Infect. Dis.* **30(3)**: 183-186.
- 289) **Masake, R. A., P. A. Majiwa, S. K. Moloo, J. M. Makau, J. T. Njuguna, M. Maina, J. Kabata, O. K. ole-MoiYoi and V. M. Nantulya.** 1997. Sensitive and specific detection of *Trypanosoma vivax* using the polymerase chain reaction. *Exp. Parasitol.* **85(2)**: 193-205.
- 290) **Corral, R. S., J. Altcheh, S. R. Alexandre, S. Grinstein, H. Freilij and A. M. Katzin.** 1996. Detection and characterization of antigens in urine of patients with acute, congenital and chronic Chagas' disease. *J. Clin. Microbiol.* **34(8)**: 1957-1962.
- 291) **Avila, H., A. M. Goncalves, N. S. Nehme, C. M. Morel and L. Simpson.** 1990. Schizodeme analysis of *Trypanosoma cruzi* sticks from South and Central America by analysis of PCR-amplified minicircle variable region sequences. *Mol. Biochem. Parasitol.* **42**: 175-188.

- 292) Avila, H. A., J. B. Pereira, O. Thiemann, E. De Paiva, W. Degrave, C. M. Morel and L. Simpson. 1993. Detection of *Trypanosoma cruzi* in blood specimens of chronic chagasic patients by polymerase chain reaction amplification of kinetoplast minicircle DNA: comparison with serology and xenodiagnosis. *J. Clin. Microbiol.* **31**: 2421-2426.
- 293) Avila, H. A., D. S. Sigman, L. M. Cohen, R. C. Millikan and L. Simpson. 1991. Polymerase chain reaction amplification of *Trypanosoma cruzi* kinetoplast minicircle DNA isolated from whole blood lysates: diagnosis of chronic Chagas' disease. *Mol. Biochem. Parasitol.* **48**: 211-222.
- 294) Diaz, C., V. Nussenzweig and A. Gonzalez. 1992. An improved polymerase chain reaction assay to detect *Trypanosoma cruzi* in blood. *Am. J. Trop. Med. Hyg.* **46**: 616-623.
- 295) Moser, D. R., L. V. Kirchoff and J. E. Donelson. 1989. Detection of *Trypanosoma cruzi* by DNA amplification using the polymerase chain reaction. *J. Clin. Microbiol.* **27**: 1477-1482.
- 296) Murthy, V. K., K. M. Dibbern and D. A. Campbell. 1992. PCR amplification of mini-exon genes differentiates *Trypanosoma cruzi* from *Trypanosoma rangeli*. *Mol. Cell. Probes.* **6**: 237-243.
- 297) Requena, J. M., A. Jimenez-Ruiz, M. Soto, M. C. Lopez and C. Alonso. 1992. Characterization of a highly repeated interspersed DNA sequence of *Trypanosoma cruzi*: its potential use in diagnosis and strain classification. *Mol. Biochem. Parasitol.* **51**: 271-280.
- 298) Russomando, G., A. Figuerdo, M. Almiron, M. Sakamoto and K. Morita. 1992. Polymerase chain reaction-based detection of *Trypanosoma cruzi* DNA in serum. *J. Clin. Microbiol.* **30**: 2864-2868.
- 299) Sturm, N. R., W. Degrave, C. Morel and L. Simpson. 1989. Sensitive detection and schizodeme classification of *Trypanosoma cruzi* cells by amplification of kinetoplast minicircle DNA sequences: use in diagnosis of Chagas' disease. *Mol. Biochem. Parasitol.* **33**: 205-214.
- 300) Suzuki, Y., M. A. Orellana, R. D. Schreiber and J. S. Remington. 1988. Interferon-gamma: the major mediator of resistance against *Toxoplasma gondii*. *Science.* **240**: 516-518.
- 301) Sher, A., E. Y. Denkers and R. T. Gazzinelli. 1995. Induction and regulation of host cell-mediated immunity by *Toxoplasma gondii*. *Ciba Found. Symp.* **195**: 95-104.
- 302) Gomez Marin, J. E., J. M. Pinon, A. Bonhomme and M. Guenounou. 1997. Does human toxoplasmosis involve an imbalance in T1/T2 cytokines. *Med. Hyp.* **48**: 161-169.
- 303) Bulmer, J. N., J. Smith, L. Morrison and M. Wells. 1988. Maternal and fetal cellular relationship in the human placental basal plate. *Placenta.* **9**: 237-246.

- 304) **Lee, H., C. D. Gregory, G. B. Rees and P. R. Golding.** 1987. Cytotoxic activity and phenotypic analysis of natural killer cells in early normal human pregnancy. *J. Reprod. Immunol.* **12**: 35-47.
- 305) **Formby, B.** 1995. Immunologic response in pregnancy. Its role in endocrine disorders of pregnancy and influence on the course of maternal autoimmune disease. *Endocrinol. Met. Clin. North Am.* **24**: 187-205.
- 306) **Barker, K. F., and R. E. Holliman.** 1992. Laboratory techniques in the investigation of toxoplasmosis. *Gen. Med.* **68(1)**: 55-59.
- 307) **De Jonge, N., Y. E. Fillié and A. M. Deedler.** 1987. A simple and rapid treatment (TCA precipitation) of serum samples to prevent non-specific reactions in the immunoassay of a proteoglycan. *J. Immunol. Methods.* **99**: 195-197.
- 308) **Siegel, J. P. and J. S. Remington.** 1983. Circulating immune complexes in toxoplasmosis: detection and clinical correlates. *Clin. Exp. Immunol.* **52**: 157-163.
- 309) **van Knapen, F., S. O. Panggabean and J. van Leusden.** 1985. Demonstration of *Toxoplasma* antigen containing complexes in active toxoplasmosis. *J. Clin. Microbiol.* **22(4)**: 645-650.
- 310) **Lindenschmidt, E. G.** 1985. Enzyme-linked immunosorbent assay for detection of soluble *Toxoplasma gondii* antigen in acute-phase toxoplasmosis. *Eur. J. Clin. Microbiol.* **4(5)**: 488-492.
- 311) **Ware, P. L. and L. H. Kasper.** 1987. Strain-specific antigens of *Toxoplasma gondii*. *Infect. Immun.* **55(3)**: 778-783.
- 312) **Kasper, L. H.** 1989. Identification of stage-specific antigens of *Toxoplasma gondii*. *Infect. Immun.* **57(3)**: 668-672.
- 313) **Suzuki, Y., K. Joh, M. A. Orellana, F. K. Conley and J. S. Remington.** 1991. A gene(s) within the H-2D region determines the development of toxoplasmic encephalitis in mice. *Immunology.* **74**: 732-739.
- 314) **Hunter, C. A., C. W. Roberts and J. Alexander.** 1992. Kinetics of cytokine mRNA production in the brains of mice with progressive toxoplasmic encephalitis. *Eur. J. Immunol.* **22**: 2317-2322.
- 315) **McLeod, R., E. Skamene, C. R. Brown, P. Eisenhauer and D. G. Mack.** 1989. Genetic regulation of early survival and cyst number after peroral *Toxoplasma gondii* infection of AXB/BXA recombinant inbred and B10 congenic mice. *J. Immunol.* **143**: 3031-3034.
- 316) **Suzuki, Y. and J. Koh.** 1994. Effect of the strain of *Toxoplasma gondii* on the development of toxoplasmic encephalitis in mice treated with antibody to interferon-gamma. *Parasitol. Res.* **80**: 125-130.
- 317) **Derouin, F. and Y. J. F. Garin.** 1991. *Toxoplasma gondii*: blood and tissue kinetics during acute and chronic infections in mice. *Exp. Parasitol.* **73**: 460-468.

- 318) Sumyuen, M. H., Y. J. F. Garin, and F. Derouin.** 1995. Early kinetics of *Toxoplasma gondii* infection in mice orally infected with cysts of an avirulent strain. *J. Parasitol.* **81**: 327-329.
- 319) Kasper, L. H., M. S. Bradley and E. R. Pfefferkorn.** 1984. Identification of stage specific sporozoite antigens of *Toxoplasma gondii* by monoclonal antibodies. *J. Immunol.* **132**: 443-449.
- 320) Kasper, L. H. and P. L. Ware.** 1985. Recognition and characterization of stage-specific oocyst/sporozoite antigens of *Toxoplasma gondii* by human antisera. *J. Clin. Invest.* **75**: 1570-1577.