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STUDIES ON THE EPIDEMIOLOGY OF TOXOPLASMOSIS IN SOUTH AFRICA

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Dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of

Master of Science in Medicine.

Johannesburg, 2009

I, Kesenthri Kistiah, declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

This work was done under ethical clearance from the Human Research Ethics Committee (Medical) of the University of the Witwatersrand (Clearance Certificates M070637 and M070347) and the Animal Ethics Screening Committee (Clearance Certificate 2008/50/01)

Kesenthri Kistiah

January 2009

To my loving parents,

Alec and Kanthi Kistiah

ABSTRACT

Toxoplasmosis is an infection of vertebrates caused by the obligate intracellular protozoan parasite, *Toxoplasma gondii*. It is one of the most common parasitic diseases of humans, infecting approximately one third of the world's population. It is a significant cause of congenital disease and an important opportunistic pathogen which has become an increasing problem worldwide due to the AIDS epidemic. There is limited historical information about the disease in South Africa. More knowledge is needed at a regional level to properly consider solutions aimed at reducing the risk for this disease. The seroprevalence of *T. gondii* in samples of selected populations at risk, namely HIV-positive individuals and a more general population sample biased towards pregnant women, was therefore investigated and found to be 9.8% (37/376) and 6.4% (32/497) in the respective samples. The Pastorex Toxo latex agglutination test was evaluated and found to be a cheap, reliable method to screen for *T. gondii* exposure. PCR-based diagnostics were developed for direct diagnosis on tissue samples. Rodent *T. gondii* infection prevalence was investigated, but did not yield any positive results. This study helped to answer questions relating to the seroprevalence and diagnosis of *T. gondii* in South Africa. Many questions still remain to be answered, however to fully understand the impact of this parasite in our country.

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ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
ARV	Antiretroviral
CD4	Cluster of differentiation 4
CFT	Complement fixation test
CSF	Cerebrospinal fluid
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
HFF	Human foreskin fibroblast cells
IFA	Indirect fluorescent antibody assay
IFL	Indirect immunofluorescence assay
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
LDM	Long distance migration

$MgCl_2$	Magnesium chloride
NPV	Negative predictive value
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PPV	Positive predictive value
RPM	Revolutions per minute
SAG2	Surface antigen two
SAG3	Surface antigen three
TBE	Tris/borate/EDTA
TE	Toxoplasmic encephalitis
T. gondii	*Toxoplasma gondii*
TMP-SMX	Trimethoprim plus sulfamethoxazole
WHO	World Health Organization
µm	Micrometer
µl	Microlitre

CHAPTER 1

INTRODUCTION

1.1. History

Toxoplasmosis is an infection of vertebrates caused by the obligate intracellular protozoan parasite, *Toxoplasma gondii* (Tenter *et al*., 2000). *T. gondii* is one of the most common parasites of humans worldwide, infecting approximately one third of the world's population (Tenter *et al*., 2000). It is a facultative heteroxenous parasite whose definitive hosts are members of the family Felidae, but is capable of infecting mammals, birds and reptiles as intermediate hosts. Its broad host range, high infection rate, worldwide distribution and the ability to maintain a benign coexistence with its host, are features of *T. gondii* which allow it to be widely regarded as one of the most successful parasites on Earth (Carruthers, 2002).

T. gondii belongs to the Phylum Apicomplexa, class Sporozoasida and subclass Coccidiasina. The genus name '*Toxoplasma*' is derived from the Greek word 'toxon' meaning bow, which describes the crescentric shape of the tachyzoite stage, and the species name '*gondii*' from the rodent from which it was first isolated (Markus, 2003).

T. gondii was first discovered 100 years ago by Nicolle and Manceaux at the Pasteur Institute in Tunisia, in 1908. Whilst conducting leishmaniasis research on the North African rodent, *Ctenodactylus gondi,* the investigators isolated *T. gondii* merozoites from its blood, liver and spleen (Frenkel, 1973; Cox, 2002; Sukthana, 2006). Around

the same time in São Paulo, Brazil, Splendore independently described *T. gondii* in a laboratory rabbit, thus suggesting its worldwide distribution (reviewed by Frenkel, 1973).

The first human case of toxoplasmosis was described by Janků in 1923 (Cox, 2002; Sukthana, 2006). He observed tissue cysts in the retina of an 11-month-old infant in Prague (Sukthana, 2006). In 1939, Wolf *et al.* successfully isolated the parasite from tissue from a neonate with encephalitis, by animal inoculation (Cox, 2002). This served to be the first example of an organism causing disease in utero. Adult infection with the parasite was first described by Pinkerton and Henderson in 1941 and childhood infection by Sabin in 1942 (reviewed by Frenkel, 1973).

Toxoplasmic retinochoroiditis was first identified by Wilder in 1952 in the retinas of 50 human cases, and the parasite was later isolated from the eye in 1954 by Jacobs *et al*. Reactivation of a latent infection in man was described by Frenkel in 1956 (reviewed by Frenkel, 1973).

In 1954, Weinman and Chandler first investigated the possible transmission of the parasite through the consumption of undercooked meat. Evidence for this was provided by Jacobs, Remington and Melton in 1960. They found that *T. gondii* cysts from pigs, sheep and other experimental animals were resistant to proteolytic enzymes and were readily infectious when swallowed (reviewed by Frenkel, 1973).

In 1970, more than 60 years after the first description of its asexual stage in intermediate hosts, the life cycle of *T. gondii* was completely elucidated by the discovery of a sexual stage in the small intestine of cats (Tenter *et al*., 2000).

1.2. Morphology

T. gondii has three infective stages: the sporozoite stage in oocysts in faeces, the rapidly dividing tachyzoites found during an acute infection and the slowly dividing bradyzoite stage in cysts during latent infection (Peterson and Dubey, 2001; Jones *et al.*, 2001b).

Tachyzoites are crescentic in shape, and approximately 6µm long and 2 µm wide (Bhopale, 2003). They are coated by a three-unit membrane, namely a plasmalemma and an inner membrane consisting of two closely situated membranes, all of which form the pellicle (Dubey *et al.*, 1998; Bhopale, 2003). The various organelles making up the tachyzoite (Figure 1.1) include apical and polar rings, rhoptries, micronemes, conoid, subpellicular microtubules, mitochondria, micropores, smooth and rough endoplasmic reticula, Golgi complexes, ribosomes and a nucleus consisting of a chromatin mass and a nucleolus (Dubey *et al.*, 1998).

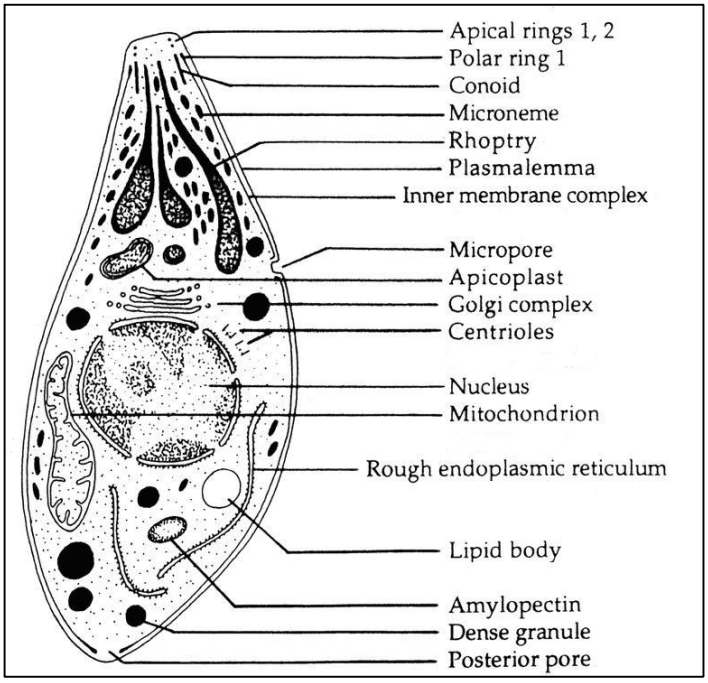


Figure 1.1: Schematic diagram showing organelles of a tachyzoite of *T. gondii*. (Adapted from Dubey *et al*., 1998.).

Tachyzoites are spread via the blood system in lymphocytes, macrophages and free in the plasma and are able to infect almost any type of tissue, especially those in the eye, central nervous system, heart, placenta and skeletal muscle (Montoya and Liesenfeld, 2004). They are capable of crossing tissue boundaries, such as the blood-brain barrier and the placenta (Carruthers, 2002). They are able to multiply rapidly by endodyogeny and such replication leads to cell necrosis when invaded cells can no longer hold these parasites and erupt. Replication of tachyzoites occurs during the first 8-12 days and accounts for the acute phase of infection (Carruthers, 2002). This stage is responsible for the clinical manifestations of the disease as it produces a strong inflammatory response (Montoya and Liesenfeld, 2004).

T. gondii induces a strong type 1 T-cell-mediated immune response, which promotes a self-limiting infection, ensuring the survival of its host and thus itself (Carruthers, 2002). As the immune response progresses, interferon-γ, secreted by antigen-specific T-cells, restricts tachyzoite replication.

Tachyzoites are sensitive to proteolytic enzymes and can therefore be destroyed during gastric digestion. The pressure of the host's immune system on the parasite stimulates the formation of cysts and causes tachyzoites to be transformed into bradyzoites, marking the beginning of the chronic phase of the infection (Carruthers, 2002; Montoya and Liesenfeld, 2004).

Bradyzoites, the slowly dividing stage of the parasite, are more resistant to proteolytic enzymes and are therefore able to cause infection if ingested as a tissue cyst by a host (Tenter *et al*., 2000). The structure of bradyzoites is illustrated in Figure 1.2.

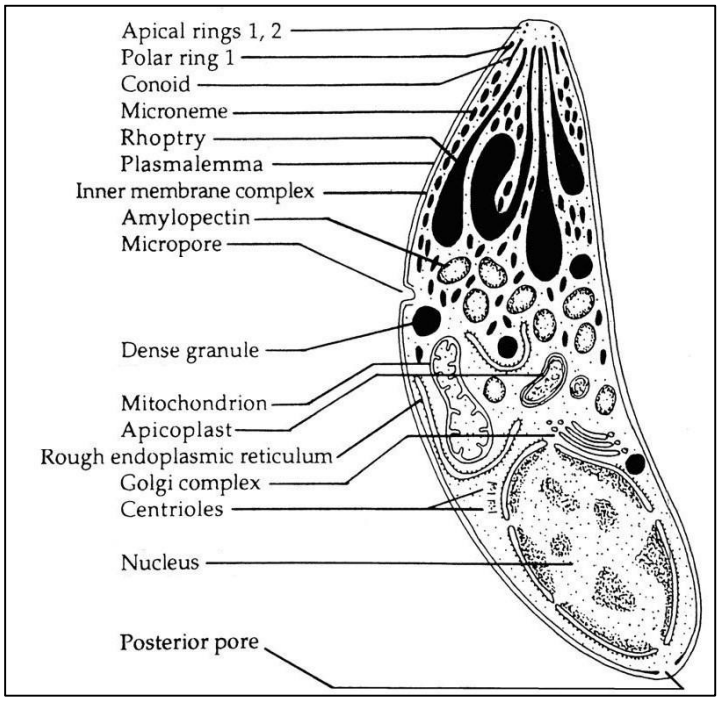


Figure 1.2: Schematic diagram showing organelles of a bradyzoite of *T. gondii*. (Adapted from Dubey *et al*., 1998.).

They are found in quiescent tissue cysts, usually in the brain where cysts are spheroidal in shape, and in muscle tissue where they are elongated (Dubey *et al.*, 1998; Roberts and McLeod, 1999). Tissue cysts are able to persist for the duration of the host's life and bradyzoites can be released from these cysts to form tachyzoites again, causing a reactivated infection in immunocompromised hosts (Montoya and Liesenfeld, 2004).

Oocysts are 10 μm to 12 μm in size and are produced within the intestine of the definitive host. Sporozoites are found within these oocysts, which are shed in the faeces of the definitive host (Peterson and Dubey, 2001).

1.3. Transmission

Transmission of the parasite can occur in several ways, as illustrated in Figure 1.3. Definitive hosts can be infected by ingestion of oocysts from the environment or cysts in prey. Transmission to humans can occur via accidental ingestion of infectious stages through faecal-oral contact, the consumption of contaminated water, fruit or vegetables, contaminated undercooked meat, via the placenta to the foetus, and rarely via organ transplantation or blood transfusion (Montoya and Liesenfeld, 2004).

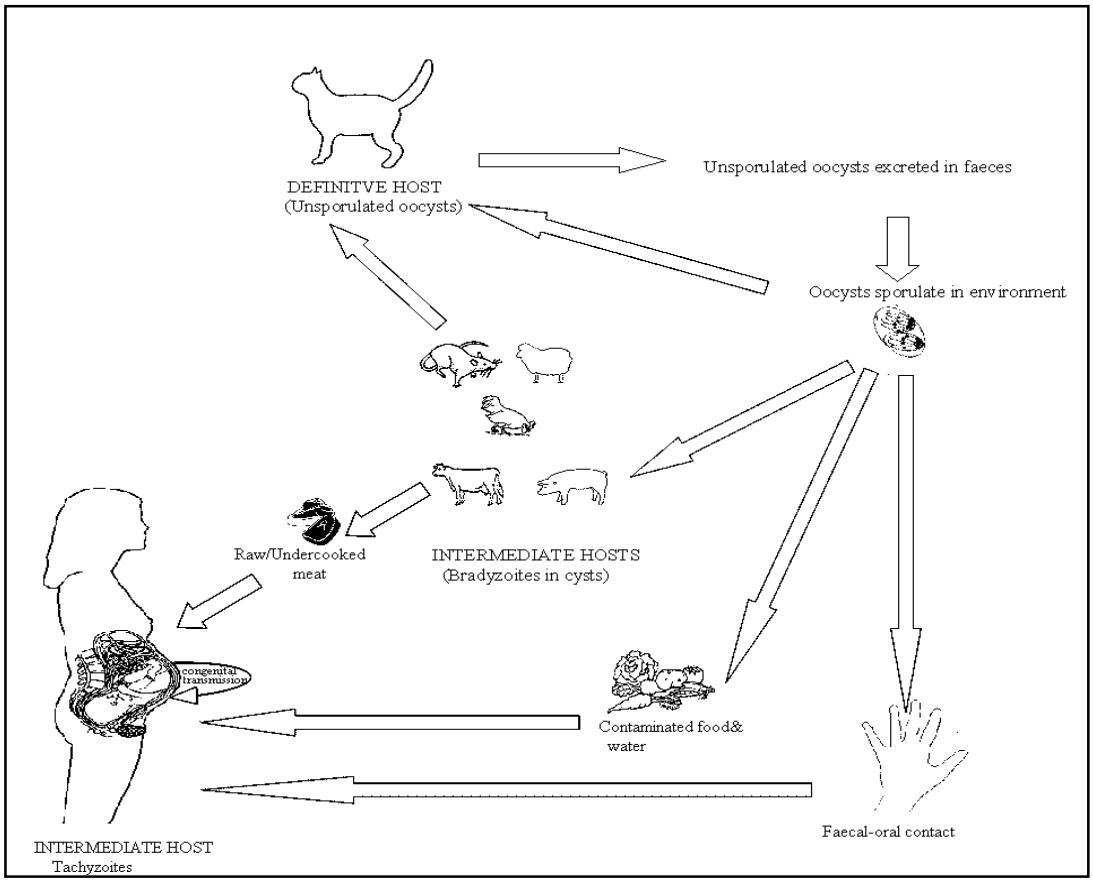


Figure 1.3: An overview of the modes of transmission of *T. gondii.*

In three large European case-control studies, undercooked meat was shown to be the largest risk factor for *T. gondii* infection. The largest of these studies showed that 30-60% of infections in pregnant women were due to the consumption of undercooked contaminated meat (Kijlstra and Jongert, 2008).

Experimental infection of cattle, sheep, pigs and goats, all of which are used for human consumption, showed that they are susceptible to *T. gondii* infection via oocysts or tissue cyst intake (Kijlstra and Jongert, 2008). As a result of infection, most animals harbour parasites in their tissues. Serological studies on cattle show prevalences that can be as high as 92%, but tissue cysts are rarely found in beef as

cattle are known to show a high resistance to *T. gondii* (Tenter *et al*., 2000). Sheep are fully susceptible to *T. gondii* infection and the consumption of mutton or lamb has therefore been identified as a significant risk for *T. gondii* infection (Kijlstra and Jongert, 2008).

Pork was generally considered a major source of infection in Europe and the USA, but recent studies on fattening pigs in the Netherlands, Austria and Germany have shown that *T. gondii* infections in pigs have dropped to less than 1% over the last ten years (Tenter *et al*., 2000). This is largely due to modern, more hygienic farming systems, as well as the increasing use of frozen meat (Kijlstra and Jongert, 2008).

Free-ranging chickens are increasingly being identified as an important source of *T. gondii* infection, especially in developing countries. Seroprevalences of up to 65% have been reported and parasite presence shown in 81% of these seropositive animals (Kijlstra and Jongert, 2008).

T. gondii-infected rats may serve as reservoirs of infection for animals such as pigs, dogs and cats, and are therefore epidemiologically potentially important in the transmission of this parasite (Dubey and Frenkel, 1998). Serological data from different countries showed that *T. gondii* prevalence in rats can be as high as 91% in the water rat, *Hydromys chrysogaster*, in Queensland, Australia. Other areas such as China, Costa Rica, Egypt, Finland, France, Germany, India, Japan, Mexico, Poland, Italy and the USA and UK have varying prevalences from 1% to 70% in different rodent species. A study in Africa by de Roever-Bonnet in 1972 showed an 8% seroprevalence in *Rattus rattus* (Dubey and Frenkel, 1998).

A rodent control campaign on three organic pig farms over a four-month period showed the disappearance of *T. gondii* from pigs in two of the three farms at the end of the campaign and subsequent reappearance in one of these farms after the rodent control campaign was stopped, thus emphasizing the role of rodents in the transmission of *T. gondii* (Kijlstra *et al.,* 2008).

The transmission of the parasite through contaminated water has generally been considered uncommon; however, the widespread infection of marine mammals indicates that that contaminated water may be a potential source of infection (Dubey, 2004). Recent outbreaks of toxoplasmosis linked to contaminated water supplies provide further evidence for this (Dubey, 2004).

1.4. Life Cycle

T. gondii has a two-stage life cycle, consisting of a sexual phase in the definitive host, and an asexual phase occurring in the intermediate host. Definitive hosts can be infected by ingestion of parasites within tissue cysts or oocysts. There are five distinct asexual phases that occur in enterocytes before gametogony begins. Microgametes fertilize macrogametes in the enterocyte forming fertilized zygotes. A wall is then laid around each zygote forming unsporulated oocysts, which are released into the intestinal lumen when enterocytes rupture. These oocysts are then released into the environment with the faeces (Peterson and Dubey, 2001). The prepatent period, which is the period between ingestion and shedding, is approximately three to ten days (Tenter *et al*., 2000; Peterson and Dubey, 2001). Cats not previously infected with *T. gondii* shed oocysts after ingestion of infectious stages, whereas cats that have

been previously infected are generally immune against renewed oocyst shedding. This immunity does not, however, continue throughout the duration of the cat's life (Petersen, 2003). Depending on the temperature, aeration and humidity of the environment, oocysts begin to sporulate within five days, dividing into two sporocysts, each containing four sporozoites. These sporulated oocysts can remain infectious for months in the environment (Peterson and Dubey, 2001).

Once ingested by an intermediate host, the outer walls of cysts or oocysts are disrupted by enzymatic degradation, releasing either bradyzoites or sporozoites into the intestinal lumen. These then actively invade surrounding cells and transform into tachyzoites. Upon entering the host cell, the parasite pulls the host cell membrane around itself and is then surrounded by a parasitophorous vacuole (Petersen, 2003; Bhopale, 2003) .Tachyzoites rapidly divide within the host cell, leading to its rupture, releasing the parasites into the blood and lymphatic system, where they are carried to other cells which they then invade, repeating this process (Bhopale, 2003; Montoya and Rosso, 2005).

1.5. Strains

T. gondii has a highly unusual population structure comprised of three clonal lineages (I, II and III). These differ in virulence and epidemiological pattern of occurrence (Montoya and Liesenfeld, 2004). Studies using murine models show that the type I strain is highly virulent and has a lethal dose of a single parasite regardless of the genetic background of the mouse. Type II and III strains have a 50% lethal dose of more than 10^3 parasites and the outcome is dependent on the genotype of the host (Mordue *et al.*, 2001). Type I and II strains have been reported in human cases, with

type I often associated with severe congenital and ocular disease, suggesting that it may be more pathogenic in humans (Mordue *et al.*, 2001). Type III has been shown to be more common in animals (Montoya and Liesenfeld, 2004).

Until recently, *T. gondii was* considered to have little genetic variability. Recent studies on *T. gondii* isolates from Brazil, however, show that they are both genetically and biologically different from those in the USA and Europe (Velmurugan *et al*., 2008). A recent study on *T. gondii* isolates from chickens in six different African countries (Nigeria, Congo, Egypt, Burkina Faso, Kenya and Mali) revealed four genotypes. Most isolates belonged to the clonal type II and III strains with one Nigerian isolate having an atypical genotype (Velmurugan *et al*., 2008). There is no information about the strains prevalent in South Africa.

1.6. Toxoplasmosis

Toxoplasmosis is a widespread disease and has been classified as the third most common cause of food-borne deaths in the USA. Over the last twenty years, it has also emerged as one of the most common opportunistic infections associated with HIV and AIDS, and is a major cause of mortality in AIDS patients in developing countries (Carruthers, 2002).

1.6.1. Clinical manifestations

The clinical manifestations of toxoplasmosis vary, depending on parasite characteristics such as virulence of the strain and inoculum size, as well as host factors such as genetic background and immune status (Montoya and Liesenfeld,

2004). Toxoplasmosis in immunocompetent individuals is typically mild or asymptomatic and usually results in life-long immunity (Tenter *et al*., 2000; Markus, 2003). Symptoms most commonly include lymphadenopathy, which may be accompanied by headache, fever, fatigue, muscular or abdominal pain as well as myocarditis, hepatitis and pulmonary necrosis (Tenter *et al*., 2000; Bhopale, 2003; Markus, 2003). However, there are cases of the infection where overtly serious clinical symptoms may occur; these include ocular toxoplasmosis, congenital toxoplasmosis, and the reactivation of a latent infection in immunocompromised individuals.

1.6.2. Ocular toxoplasmosis

T. gondii is the most common cause of retinochoroiditis in humans worldwide, accounting for 28% to 55% of all cases of posterior uveitis (Pavesio and Lightman, 1996; Lappalainen and Hedman, 2004; Vallochi *et al.*, 2005; Bonfioli and Orefice, 2005).

Retinochoroiditis commonly occurs as a result of congenital infection but can also be due to an acquired or reactivated infection (Bonfioli and Orefice, 2005; Dubey and Jones, 2008). It is usually a self-limiting disease with lesions healing within six weeks (Rothova, 1993). Active lesions usually present with a white focus of necrotizing retinochoroiditis close to old pigmented scars. These lesions are usually circular or oval in shape and they vary in size. In the congenital forms, the lesions are usually bilateral and central and in the acquired forms, the lesions are usually unilateral and solitary (Pavesio and Lightman, 1996).

Parasites reach the eye as free tachyzoites or as cysts which rupture, releasing tachyzoites. Once infected cells lyse, tachyzoites invade the retina and multiply in the surrounding cells causing an inflammatory response (Rothova, 1993; Roberts and McLeod, 1999). Clinical manifestations of acute retinochoroiditis include tearing, pain, photophobia and progressive loss of vision over time, especially when there is macular or optic nerve involvement (Pavesio and Lightman, 1996; Dubey and Jones, 2008).

1.6.3. Congenital toxoplasmosis

Toxoplasmosis is a significant cause of congenital disease. Congenital toxoplasmosis occurs in between 1 and 10 per 10 000 live births in Europe (Cook *et al.*, 2000; Tenter *et al.*, 2000; Montoya and Liesenfeld, 2004). Transplacental transmission occurs when an immunocompetent woman acquires a primary infection during pregnancy (Hegab and Al-Mutawa, 2003), or may also be due to a reactivated infection in immunocompromised women (Dubey and Jones, 2008). Primary infections acquired four to six months before conception usually result in no transplacental transmission to the foetus (Tenter *et al.*, 2000; Jones *et al.*, 2001b).

Primary infection during pregnancy may result in severe damage or death of the foetus and long-term sequelae in the child. The risk of congenital infection increases from the first trimester (10-25%) to the third trimester (60-90%) with the development of a good blood flow (Jones *et al.*, 2001b; Hegab and Al-Mutawa, 2003; Dubey and Jones, 2008). The severity of the disease, however, is the highest in the first trimester and lowest in the third trimester (Jones *et al.*, 2001b; Hegab and Al-Mutawa, 2003). Infection within the first two trimesters may result in death of the foetus in utero or

spontaneous abortion. Infection in the last trimester usually results in newborns that are asymptomatic at birth, but may develop symptoms later in life (Montoya and Liesenfeld, 2004).

Most children born with congenital toxoplasmosis are asymptomatic at birth; however, approximately 80% of them will develop neurological or ocular sequlae later in life (Rothova, 1993; Roberts and McLeod, 1999; Boyer, 2000; Jones *et al.*, 2003; Dubey and Jones, 2008). Approximately 10% of prenatal infections result in abortion or neonatal death, and 10–23% of infected newborns show clinical signs of toxoplasmosis at birth (Luft and Remington, 1992). There may be mild disease such as reduced vision, or it may cause severe abnormalities such as blindness, mental retardation and epilepsy (Tenter *et al.*, 2000). The classic triad of signs is hydrocephalus, retinochoroiditis and intracranial calcifications, and occurs in approximately 10% of all infected newborns (Tenter *et al.*, 2000).

1.6.4. Toxoplasmosis in immunocompromised hosts

Reactivated latent infections of toxoplasmosis in immunocompromised individuals, such as those with HIV/AIDS, Hodgkin's disease or those undergoing immunosuppressive therapy, can be life threatening (Tenter *et al*., 2000). It has become an increasing problem worldwide due to the AIDS epidemic. Studies prior to widespread use of antiretroviral treatment show that approximately 10% of AIDS patients in the USA and 30% in Europe die from toxoplasmosis (Luft and Remington, 1992). The brain is the most common site of infection in immunocompromised individuals. Toxoplasmic encephalitis (TE) occurs in approximately 40% of AIDS

patients worldwide (Tenter *et al*., 2000; Aoun *et al.*, 2006). Highly active antiretroviral therapy (HAART), which helps to decrease the viral load and improve $CD4^+$ T-cell counts, and prophylactic treatment against reactivation of latent *T. gondii* infections, have helped to decrease the incidence of TE (Tenter *et al*., 2000; Sukthana, 2006).

Early symptoms of TE include a persistent bilateral headache. Disease progression leads to severe manifestations such as confusion, lethargy, mental state changes, seizures, ataxia and coma, and the outcome may be fatal (Hill and Dubey, 2002; Montoya and Liesenfeld, 2004). Approximately two-thirds of all people living with HIV are in sub-Saharan Africa. According to the UNAIDS 2008 report on the global AIDS epidemic, approximately 5.7 million South Africans were living with HIV at the end of 2007. In areas such as this, where it is estimated that almost 1000 AIDS-related deaths occur every day, toxoplasmosis could theoretically pose more of a threat than almost anywhere else in the world.

1.6.5. Diagnosis

Toxoplasmosis is frequently asymptomatic and clinical manifestations, when present, are usually non-specific and mimic other infections, making definitive clinical diagnosis very difficult (Hill and Dubey, 2002; Kompalic-Cristo *et al.*, 2004). Diagnosis is usually made by immunological testing, histological identification, isolation in tissue culture, recovery of the parasite DNA by the polymerase chain reaction (PCR) or by a combination of these techniques. Cerebral toxoplasmosis can also be diagnosed using computerized tomography and magnetic resonance imaging (Hill and Dubey, 2002; Markus, 2003; Sukthana, 2006).

Serological tests are most widely used, yet they have the greatest limitations as they often provide ambiguous results (Markus, 2003; Kompalic-Cristo *et al.*, 2004). Examples include the Sabin-Feldman dye test, which is the traditional gold standard, indirect fluorescent antibody assay (IFA), complement fixation test (CFT) and the enzyme-linked immunosorbent assay (ELISA) (Hill and Dubey, 2002).

Serological tests are used to detect increased antibody levels such as IgG, IgM, IgA and IgE (Jones *et al.*, 2003). In a primary *T. gondii* infection, IgM appears a few weeks after infection, followed by IgA and IgE. These acute phase immunoglobulins peak after about two months and are usually undetectable by serological tests by six to nine months but can persist for longer periods of time (Montoya and Rosso, 2005; Sukthana, 2006). IgG, which appears after IgM, peaks after four months and persists at low levels throughout the duration of the host's life (Sukthana, 2006).

A problem with serological tests is that the detection of antibodies in immuno-compromised individuals may be difficult due to the deterioration of the immune system (Schneider *et al.,* 1992). A further problem is that IgM may persist for longer than expected periods and discrimination between recent and older infections may therefore be a problem (Ho-Yen *et al.*, 1992; Remington *et al.*, 2004). This is an important factor when diagnosing toxoplasmosis in immunocompromised individuals as the presence of IgG indicates a risk for the reactivation of a latent infection, and IgM indicates the possibility of an acute infection. In pregnant women, positive IgM results indicate the likely acquisition of infection during gestation and a positive IgG and negative IgM result indicates a previous infection (Montoya and Rosso, 2005).

Avidity tests have helped to overcome this problem as they help differentiate between recently and distantly acquired infections (Lappalainen and Hedman, 2004; Montoya and Rosso, 2005). Avidity tests are based on the fact that during acute infections, IgG antibodies bind antigen relatively weakly and therefore have a low avidity. Chronic infections, however, have more strongly-binding antibodies and therefore have a high avidity (Lappalainen and Hedman, 2004; Montoya and Rosso, 2005).

Some of these problems can be overcome with the use of PCR. This method has both advantages and disadvantages. Advantages are that the detection of nucleic acid is not affected by the condition of the immune system, it is generally more sensitive and rapid than serological tests and diagnosis can be made from biopsies, blood, cerebrospinal fluid (CSF) and amniotic fluid. Disadvantages are that false positive results due to contamination may occur, it may be too sensitive in detecting non-viable *T. gondii* remnants that do not cause disease, and may yield false negative results due to inhibition (Johnson *et al.*, 1993).

These problems with PCR can, however, be overcome and more rapid and sensitive methods are regularly being developed. These advances in PCR techniques are helping to make it an invaluable diagnostic tool.

1.6.6. Prevention and treatment

Toxoplasmosis is a curable but potentially fatal disease. To prevent infections in humans, a number of measures can be taken. Individuals should practice good hygiene. Hands should be washed thoroughly after handling meat or soil before beginning any other tasks. All fruit and vegetables must also be washed before they are to be consumed. All meat should be well cooked to a minimum temperature of 67 ºC before consuming so as to kill tissue cysts. Tissue cysts can also be killed by cooling to -13 ºC. Pregnant women should be especially careful, and should limit contact with cats, cat litter, soil and raw meat (Tenter *et al.*, 2000; Hill and Dubey, 2002).

Treatment for immunocompetent individuals is usually not necessary. Sulfadiazine plus pyrimethamine is the most commonly recommended therapy for congenital and ocular toxoplasmosis, as well as infection in immunocompromised individuals (Boyer, 2000; Hill and Dubey, 2002; Montoya and Liesenfeld, 2004). Alternative treatments for patients intolerant to sulphonamides are also used. These include clindamycin plus pyrimethamine, clarithromycin plus pyrimethamine, and atovaquone (Arens *et al.*, 2007). Co-trimoxazole (trimethoprim plus sulfamethoxazole, TMP-SMX) is used in place of sulfadiazine and pyrimethamine in South Africa as sulfadiazine has been removed from the South African market. A study by Arens *et al*. in 2007 showed that there were no differences in clinical outcome between these two regimens, but co-trimoxazole was better tolerated (Arens *et al.,* 2007). Maintenance therapy of half the dose of the therapeutic drugs is usually administered

as the drugs are not effective against tissue cysts, which could reactivate (Sukthana, 2006).

1.7. Epidemiology

T. gondii has a worldwide distribution, but its prevalence varies greatly. Table 1.1, which summarises seropositivity rates in Europe, the Americas and Southeast Asia, shows this distinct geographical variation.

Table 1.1: Seropositivity rates for toxoplasmosis in Europe, the Americas and Southeast Asia

Continents and countries	Year	Seropositivity (%)
Western Europe		
Austria	1998	43
Belgium	1997	50
France	2001	Up to 75
Germany	2004	26–54
Italy	2001	18–60
The Netherlands	2004	40.5
Spain	2004	28.6
Switzerland	1995	46
UK	1992	23–33
Scandinavia		
Denmark	1999	27.8
Finland	1995	20.3
Norway	1998	10.9
Sweden	2001	14.0–29.4
Central and Eastern Europe		
Croatia	2000	38.1
Poland	2001	46.4–58.5

Continents and countries	Year	Seropositivity (%)
Slovenia	2002	34
Yugoslavia	1998	57–93
North America		
USA	2004	16–40
Central America		
Costa Rica	1996	76
Cuba	1993	60
Mexico	2001	35
Panama	1988	90 (at 60 years of age)
South America		
Argentina	2001	72
Brazil	2001	59
West Indies	1991	29.7
Southeast Asia		
Indonesia	2000	58
Malaysia	2004	44.8
Thailand	1992, 1997, 2000, 2001	2.3–21.9

(From: Sukthana, 2006).

Besides varying between different continents and countries, *T. gondii* prevalence also varies within the same country. A study by Jones *et al.* (2001a), showed that the seroprevelence in the USA varied from 29.2% in the northeast to 20.5% and 17.5% in the south-midwest and west respectively.

There is little known about *T. gondii* prevalence in Africa. Table 1.2 shows the results of some studies carried out on this continent.

Table 1.2: Seropositivity rates in Africa

Country	**Year**	**Study population**	**Seroprevalence (%)**	**Reference**
South Africa and Botswana	1978	See details in text (below)	20 11	Jacobs and Mason, 1978
Kenya	1991	HIV-positive and -negative adults	54	Brindle *et al*., 1991
Sudan	1991	Apparently healthy individuals	41.7	Abdel-Hameed, 1991
Zambia	1991	HIV-positive and -negative adults	7	Zumla *et al*., 1991
Cameroon	1992	Pregnant women	77.1	Ndumbe *et al*., 1992
Senegal	1993	Women	40.3	Diallo *et al*., 1996
Republic of Benin	1995	Pregnant HIV-negative women	53.6	Rodier *et al*., 1995
Tanzania	1995	Pregnant women	35	Doehring *et al*., 1995
Ethiopia	1998	HIV-positive and -negative adults	80	Woldemichael *et al*., 1998
Burkina Faso	2004-2005	Pregnant women	25.3	Simpore *et al*., 2006
Uganda	2006	HIV-positive adults	54	Lindström *et al.*, 2006

Figure 1.4 illustrates that the geographical variation observed in other parts of the world is very similar in Africa.

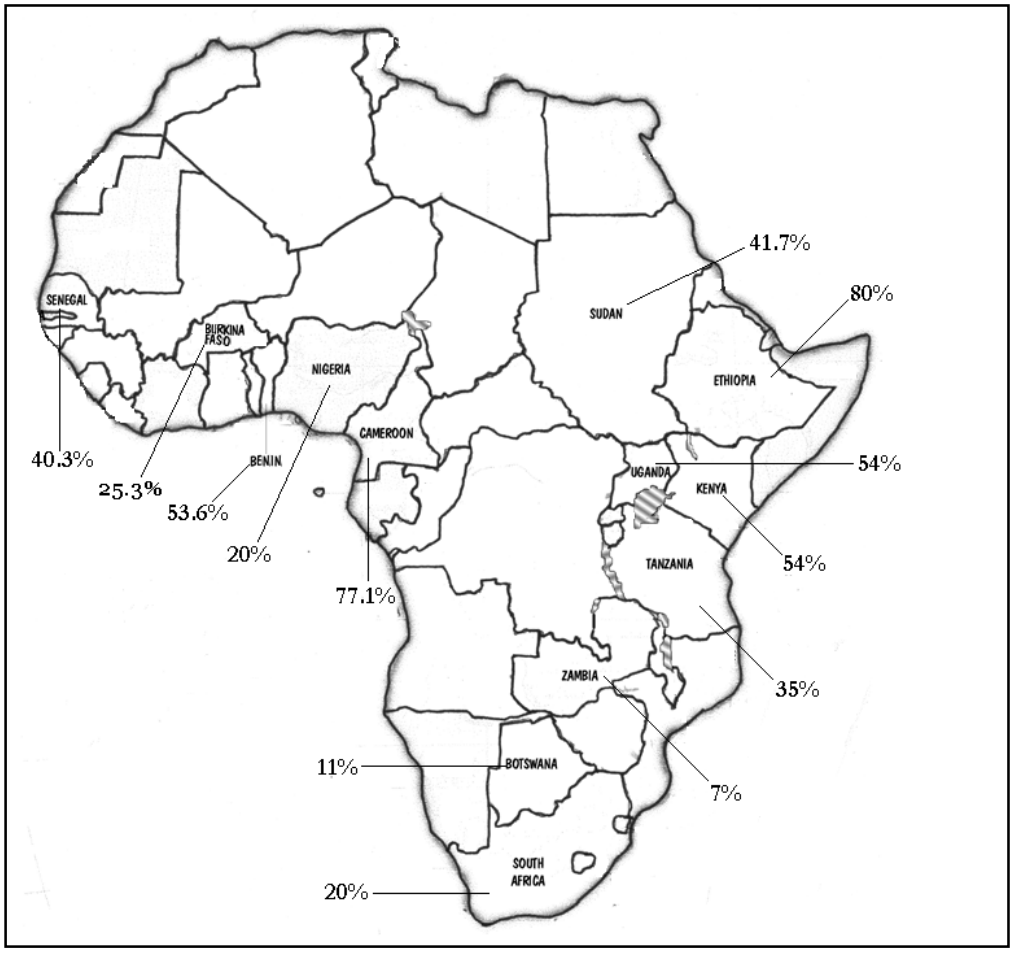


Figure 1.4: Variation in *T. gondii* seroprevalence in Africa.

There is limited historical information about the disease in South Africa. A study by Jacobs and Mason in 1978, examined the seroprevalence of *T. gondii* in four regions of southern Africa (Natal, Western Cape, Eastern Cape and the combined South West Africa and Botswana region) amongst five different ethnic groups (blacks, whites, coloureds, Indians and San). An overall prevalence of 20% was found amongst these

different regions and population groups. The prevalence amongst specific areas and ethnic groups is summarized in Table 1.3.

Table 1.3: Prevalence amongst different ethnic groups and areas

	White (%)	**Black (%)**	**Coloured (%)**	**Indian (%)**	**San (%)**	**Total (%)**
Eastern Cape (Port Elizabeth)	17	30	30	-	-	24
Western Cape (Cape Town)	11	-	23	-	-	18
Natal (Durban)	24	34	-	33	-	30
South West Africa and Botswana	12	27	-	-	9	11
Total (%)	16	31	26	33	9	20

(From: Jacobs & Mason, 1978.)

The highest prevalence of 30 % was found in Natal, and the lowest in the South West Africa and Botswana (11%). Indians and blacks had the highest prevalences ethnically, followed by the coloured, white and San populations respectively.

A similar study by Mason *et al.* in 1974 showed an overall prevalence of 37% in the Transvaal Province of South Africa. The highest prevalence in this area was amongst the Indian population (58%), followed by coloured (43%), white (33%) and black (29%) populations.

In 1992, Schneider *et al.* investigated the *T. gondii* prevalence in women of different ethnic groups in Durban, KwaZulu-Natal Province. An overall prevalence of 31.3% was found and the highest prevalence here was amongst the black population (46.2%), followed by the Indian (36.9%), coloured (28.3%) and white (12.5%) populations.

A study on toxoplasmosis and HIV infection in South Africa by Sonnenberg *et al.* (1998), was carried out at the Gold Fields West Mine Hospital. Patients originated from several different home regions, such the Eastern Cape and KwaZulu-Natal Provinces, Lesotho and Mozambique, and an overall prevalence of 24.6% was found.

The most recent study from the Gauteng Province by Hari *et al.* in 2007 was conducted at the Helen Joseph Hospital in Johannesburg, and investigated *T. gondii* seroprevalence in 307 consecutive HIV-infected medical inpatients. All patients were black, antiretroviral naïve and did not receive toxoplasmosis prophylaxis. An overall prevalence of 8% was found, with only two patients showing clinical manifestations.

Detailed recent demographic data of groups at risk is missing and more knowledge is needed at a regional level, e.g. seroprevalence of infection, to implement solutions aimed at reducing risk for this disease in South Africa.

1.8. Climate and topography of South Africa

South Africa is located on the southernmost tip of Africa. The country's coastline stretches more than 2 500km from the border of Mozambique along the Indian Ocean,

around the tip of Africa and up towards Namibia along the Atlantic Ocean. It consists of a low-lying narrow coastal zone, mountainous escarpments and a high inland plateau (Brand South Africa, 2008).

The country has nine provinces and five climatic regions. A temperate sub-tropical central region is characterised by hot wet summers and cold, dry winters, with frost and snow occurring in the mountains. The subtropical region is located on the eastern border and coastline of the country, where summers are warm and humid and winters are mild. Rain falls throughout the year with summer being the wettest season. The Mediterranean climatic region experiences hot, dry summers with occasional rainstorms and cool, wet winters. This region extends only over a narrow coastal area near Cape Town. The semi-desert region experiences high temperatures and low rainfall, which usually falls during the summer months, and the desert region experiences very little rain and very high temperatures inland (Brand South Africa, 2008).

Much of South Africa is classified as semi-arid. The cold Benguela current from the west coast and the warm Agulhas current from the east coast have a major effect on the country's climate. The high evaporation rate of the Agulhas current provides significant rainfall on the east, while the Benguela current retains its moisture causing desert conditions in the west. Mean temperatures vary from 15 ºC to 30 ºC during the summer to 10 ºC to 25 ºC during the winter across different regions inland (Brand South Africa, 2008).

Figures 5.1-5.3 show the mean temperature, rainfall and humidity for three of South Africa's major cities: Cape Town, Durban and Johannesburg.

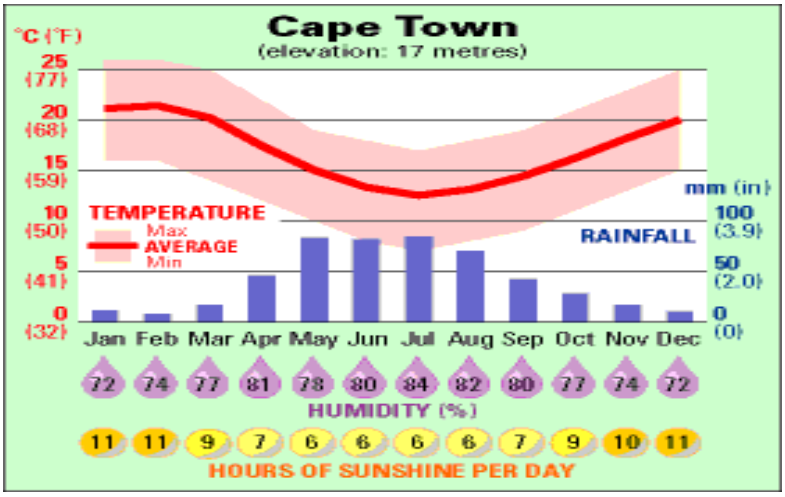


Figure 1.5: Average temperature, humidity and rainfall of Cape Town.

(Adapted from: World Travel Guide. Nexus Business Media. http://www.worldtravelguide.net/country/255/climate/Africa/South-Africa.html)

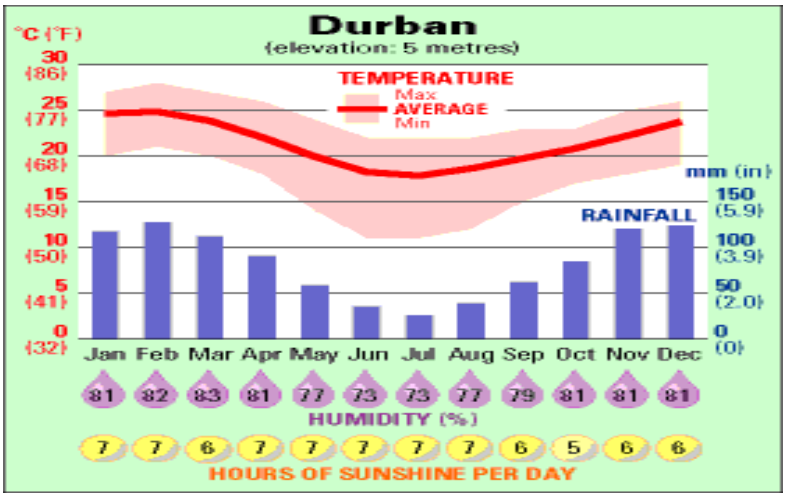


Figure 1.6: Average temperature, humidity and rainfall of Durban .

(Adapted from: World Travel Guide. Nexus Business Media. http://www.worldtravelguide.net/country/255/climate/Africa/South-Africa.html)

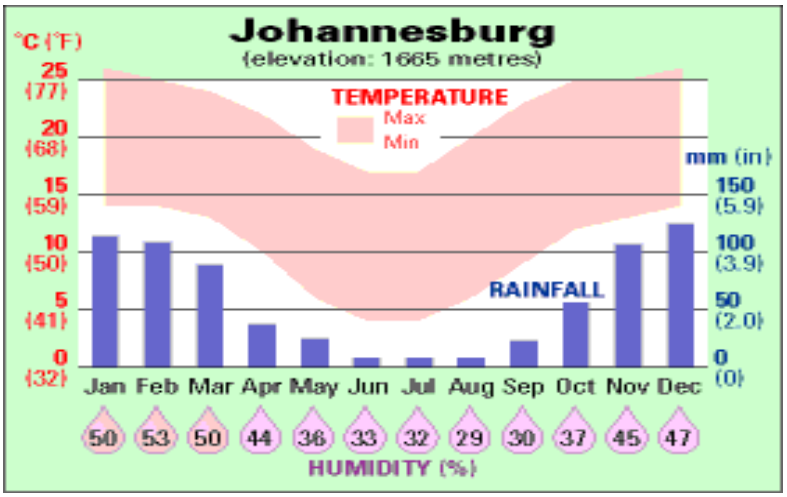


Figure 1.7: Average temperature, humidity and rainfall of Johannesburg.
(Adapted from: World Travel Guide. Nexus Business Media.
http://www.worldtravelguide.net/country/255/climate/Africa/South-Africa.html)

The city of Johannesburg has an altitude of approximately 1694 m, a mean temperature of 20 ºC and a mean rainfall of 760 mm. Humidity is generally low and there are no subtropical extremes of heat due to its high altitude. Winters are generally very cold, but snow is rare (Brand South Africa, 2008).

1.9. Aims and Objectives

The general aim of this study was to obtain a better understanding of the epidemiology of toxoplasmosis in South Africa. The specific objectives were :

1. To establish PCR-based diagnostics for toxoplasmosis using a published nested PCR method (Burg *et al.*,1989) and to determine rodent toxoplasmosis prevelance across four sites in South Africa.

2. To characterize prevalent *Toxoplasma* strains using rodent organ material. Types I, II and III were identified by a nested PCR reaction (Grigg *et al.*, 2001), amplifying a SAG3 genetic marker and subsequent digestion with restriction enzymes NciI or BcnI.

3. To conduct a retrospective human serosurvey, using a bank of residual serum, targeting mainly, but not exclusively, pregnant women and children, for evidence of toxoplasmosis, to establish prevalence rates across all population groups.

4. To conduct a prospective human serosurvey in HIV-positive patients compared to HIV-negative controls.

5. To compare a standard enzyme immunoassay (EIA) serological test with a simple, cheap latex agglutination test, which, if validated, would make screening for *Toxoplasma* exposure more accessible for the general population.

CHAPTER 2

MATERIALS AND METHODS

2.1. HIV-Positive prospective serosurvey

A prospective human serosurvey for toxoplasmosis was conducted at the HIV clinic at the Chris Hani Baragwanath Hospital in Soweto, Johannesburg. Patients enrolled in the antiretroviral (ARV) treatment programme were invited to participate. Patients were recruited at the time of collection of blood for routine investigations and sampling was done on a convenience basis. Once informed consent was given, two tubes of blood (1 EDTA-anticoagulated and 1 clotted blood) were drawn by phlebotomists at the clinic, for this study (Appendix A, pp.60-61). Ethical clearance was granted by the Human Research Ethics Committee, University of the Witwatersrand. (Protocol Number: M070637), (Appendix A, p. 62).

The required minimum sample size, based on a mean toxoplasmosis prevalence of 30% (from published South African data), taking the maximum acceptable difference between the population and the sample rate to be 4%, with a 95% confidence level, is 504, but the final number tested was 376 due to the limited amount of time available to collect samples.

Samples were then taken to the Parasitology Reference Unit at the National Institute for Communicable Diseases (NICD), which is one of the host laboratories for this study. Samples were centrifuged at 3000 revolutions per minute (RPM) for 4.5 minutes and serum was collected and aliquoted. Aliquots were labelled with sample

numbers corresponding to the information sheets and consent forms filled in by patients, so that patient identity was kept confidential.

Aliquots were taken to Department of Parasitology, Mycology and Environmental Microbiology at the Swedish Institute for Infectious Disease Control (SMI), Stockholm, Sweden, which was also a host laboratory, where all tests were carried out by the principal investigator.

Using the Pastorex Toxo latex particle agglutination test, serum samples were tested in duplicate according to the package protocol (Pastorex TOXO, package insert; Bio-Rad, France; 2006), with the operator blinded to corresponding results. This technique utilises soluble membrane and cytoplasmic antigens bound to latex particles, which agglutinate when specific antibodies (IgM and/or IgG) are present in the samples. A positive reaction is characterised by the formation of red aggregates on a green background, whereas in a negative reaction, the suspension remains brown and homogenous. Indeterminate results were categorized as those which showed a slight green background or red aggregates as well as a brown suspension. Positive and negative serum controls were included on every card.

All discrepant, positive and indeterminate results were then retested and titrated using the BioMèrieux Toxo-Screen DA test, which was the reference test for this part of the study as it corresponds to the Sabin-Feldman test, the traditional gold standard. The Biomèrieux Toxo-Screen DA detects *Toxoplasma* IgG antibodies in human serum by direct agglutination. The principle of this test is that formalin-treated *Toxoplasma* zoites agglutinate in the presence of diluted serum which contains specific IgG

antibodies (Toxo-Screen DA, package insert; BioMèrieux SA, France; 2006). Once again samples were tested in duplicate according to the package insert, with the operator blinded to corresponding results. Positive, negative and PBS controls were included in every experiment.

The discrepant, positive and indeterminate Pastorex results were also retested using indirect immunofluorescence (IFL) according to a specific protocol (Appendix B, p.65). Diluted patient serum samples were added to wells containing *Toxoplasma* tachyzoites and allowed to incubate. During this stage, any anti-*Toxoplasma*-specific antigens present in the serum bind specifically to the parasites. A second incubation with rabbit anti-human antibodies conjugated with fluorescein isothiocyanate (FITC) diluted in Evans blue detect any human antibodies present from the first incubation. Evans blue helps to create a dark background for viewing slides. Positive and negative controls, as well as a conjugate control of PBS were included with every experiment.

All BioMèrieux test-negative results were further tested for IgM using an IFL because the BioMèrieux test only detects IgG whereas Pastorex test detects both IgG and IgM.

2.2. HIV-negative controls

HIV-negative controls were obtained from the HIV diagnostic virology laboratory at the NICD. These residual human sera, previously collected under an antenatal survey programme, served as appropriate controls. They matched the test group as they were both collected in the Gauteng area from populations with similar living conditions and expected rates of exposure. Samples were identifiable only by number and confidentiality was thus ensured. Ethical approval was granted by the Human Research Ethics Committee of the University of the Witwatersrand. (Protocol Number: M 070637) (Appendix A, p.62).

Three hundred and seventy-six controls were tested in an identical manner to the HIV-positive specimens. All samples were tested in duplicate in a blinded manner using the Pastorex-Toxo agglutination test. Thereafter the BioMèrieux Toxo-Screen DA test was used to confirm all positive and indeterminate results. Testing with IFL was not carried out at this stage, as it was unlikely that IgM would be present in many of these specimens.

2.3. Retrospective serosurvey

The retrospective serosurvey involved the use of serum samples, mainly but not exclusively targeting pregnant women and children, randomly selected from a residual serum bank of 7000 specimens that had been collected for a rubella prevalence study. The age spectrum of selected subjects was wide, but the majority were young adults. (Protocol number: M070347), (Appendix A, p.63). The required minimum sample size, once again based on a mean toxoplasmosis prevalence of 30%,

taking the maximum acceptable difference between the population and the sample rate to be 4%, with a 95% confidence level, was 504. Due to availability of samples, however, only 497 were used.

Samples were sent to the Department of Immunology at the National Health Laboratory Service (NHLS), where the Axsym Toxo IgG (Abbott) automated enzyme immunoassay (EIA) was used to assay IgG antibodies and was the reference method for this part of the study. This technique utilises *Toxoplasma* antigens bound to microparticles, which are mixed with the serum sample. Specific antigen-antibody complexes are immobilised on a glass fibre matrix, and enzyme-linked antihuman globulin is used in a detection system, as in a normal plate EIA. Standardised calibrators (referenced to WHO standards) are used to produce a calibration curve against which the samples are read, to give a quantitative anti-*Toxoplasma* antibody concentration (Axsym test information; Abbott Laboratories, USA; 2005).

The Pastorex Toxo latex particle agglutination test (Bio-Rad, France), described earlier, was used on the same specimens, with the operator blinded to corresponding EIA results.

2.4. Prevalence in Rodents

In order to determine *T. gondii* prevalence in rodents as well as to establish PCR- based diagnostics for toxoplasmosis, 100 rodent spleen specimens were retrospectively sampled (as residual specimens from a different study) from each of three sites, namely, Port Elizabeth, Limpopo Province, and Durban. Twenty-five

rodent heart and brain samples were prospectively collected from the Johannesburg area. Ethical approval was granted by the Animal Ethics Screening Committee, University of the Witwatersrand (Clearance number: 2008/50/01), (Appendix A, p.64).

The required minimum sample size, based on the fact that the background *Toxoplasma* prevalence in rodents is unknown (and therefore assumed to be 50%), and with 95% confidence of being within 5% of the true prevalence, was calculated to be 384; however, due to problems with the availability of new material, only 325 samples could be collected.

Samples from Durban were mainly of the *Rattus norvegicus* species. *Rhabdomys pumilio* was most common in the sample group from Port Elizabeth and *Rattus rattus* was most common in Mapate. Samples from Johannesburg were not speciated.

DNA was extracted from the tissue according to the QIAgen tissue protocol, using a QIAamp DNA mini kit (Qiagen Inc. Valencia, C.A, USA), (Appendix B, p.66-67). Extracted DNA was then tested using a polymerase chain reaction (PCR) technique.

A nested PCR based on the identification of the 35-fold repetitive B1 gene, as described by Burg *et al.* (1989), was validated using known *Toxoplasma*-positive and negative specimens, checking for a predicted PCR band size of 97 base pairs. Once it was validated, all extracted DNA was tested according to a specific protocol (Appendix B, pp.68-70) using oligonucleotide primers, *Toxoplasma* B1 (5'- GGA ACT GCA TCC GTT CAT GAG -3'), *Toxoplasma* B2 (5'- TCT TTA AAG CGT

TCG TGG TC -3'), *Toxoplasma* B3 (5'- TGC ATA GGT TGC AGT CAC TG -3'), and *Toxoplasma* B4 (5'- GGC GAC CAA TCT GCG AAT ACA CC-3').

The PCR reaction mix and cycling parameters are described in Appendix B, pp.68-70.

Gel electrophoresis using a 2% agarose gel, was performed as described in Appendix B, pp.71-72, to identify the 97 base pair product.

DNA and PCR reagent controls were included in each experiment to monitor for possible contamination. Furthermore, DNA extraction, PCR reaction mix preparation, DNA addition, PCR amplification and gel electrophoresis were done in separate one-way traffic-controlled rooms to minimize the risk of contamination.

All results with irregular bands from the previous PCR method were retested using a nested PCR reaction as described by Grigg *et al.* (2001), amplifying the SAG3 gene. Once again, this method was first validated using known positive and negative specimens before testing was carried out according to a set protocol (Appendix B, pp.73-75) using oligonucleotide primers Primer 1 (5'- CAA CTC TCA CCA TTC CAC CC -3'), Primer 2 (5'- GCG CGT TGT TAG ACA AGA CA -3'), Primer 3 (5'- TCT TGT CGG GTG TTC ACT CA-3') and Primer 4 (5'- CAC AAG GAG ACC GAG AAG GA-3'). The PCR reaction mix and cycling parameters are described in Appendix B, pp.73-75.

DNA and PCR reagent controls were included in each experiment to monitor for possible contamination. Gel electrophoresis using a 2% agarose gel was used to identify a 226 base pair product.

The method of enzyme digestion of the SAG3 gene with restriction enzymes NciI and BcnI to identify different strain types was validated using known type I, II and III *Toxoplasma* strains. Enzymes cut the amplified sequence of type I parasites twice, type III once and did not cut type II parasites (Appendix B, p.76).

2.5. Toxoplasma cultivation

T. gondii parasites were cultivated in cell culture and used as positive DNA controls in PCR. Tachyzoites were fixed onto slides for use in IFL.

Flasks containing confluent human foreskin fibroblast cells (HFF) were infected with freshly lysed *Toxoplasma* tachyzoites, RH-LDM strain (Barragan and Sibley, 2002). Flasks were then incubated until cells lysed and parasites were collected in sediment as described in Appendix B, pp.77-78.

DNA was extracted once again using the QIAGEN DNA mini kit and used as positive PCR controls. Washed *Toxoplasma* tachyzoites were distributed on 8-well Teflon-coated microscope slides, left to air dry and then fixed in cold acetone for 20 minutes and stored frozen until used for IFL.

2.6. Data analysis

Data analysis was carried out using a statistical software package, STATISTICA (StatSoft, USA).

CHAPTER 3

RESULTS

3.1. Prevalence of anti-*T. gondii* antibodies in HIV-positive and HIV-negative patients

Thirty-seven of 376 HIV-positive patients possessed antibodies to *T. gondii*, and the overall prevalence in this sample was found to be 9.8%. Forty-eight of the 376 HIV-negative controls possessed antibodies to *T. gondii*. An overall prevalence of 12.8% in this sample was calculated. IFL showed no IgM positive samples.

Comparison of anti-*T. gondii*-antibody presence in HIV-positive patients and HIV-negative controls are summarized in Table 3.1 and Figure 3.1. Statistical analysis using the Chi-squared test gave a p-value < 0.05, showing a statistical difference between these two groups.

Table 3.1: 2x2 table showing anti-*T. gondii*-antibody presence in HIV-positive patients and HIV-negative controls

Presence of antibodies				
HIV Status		+	-	Total
	+	37	339	376
	-	48	328	376
	Total	85	667	752

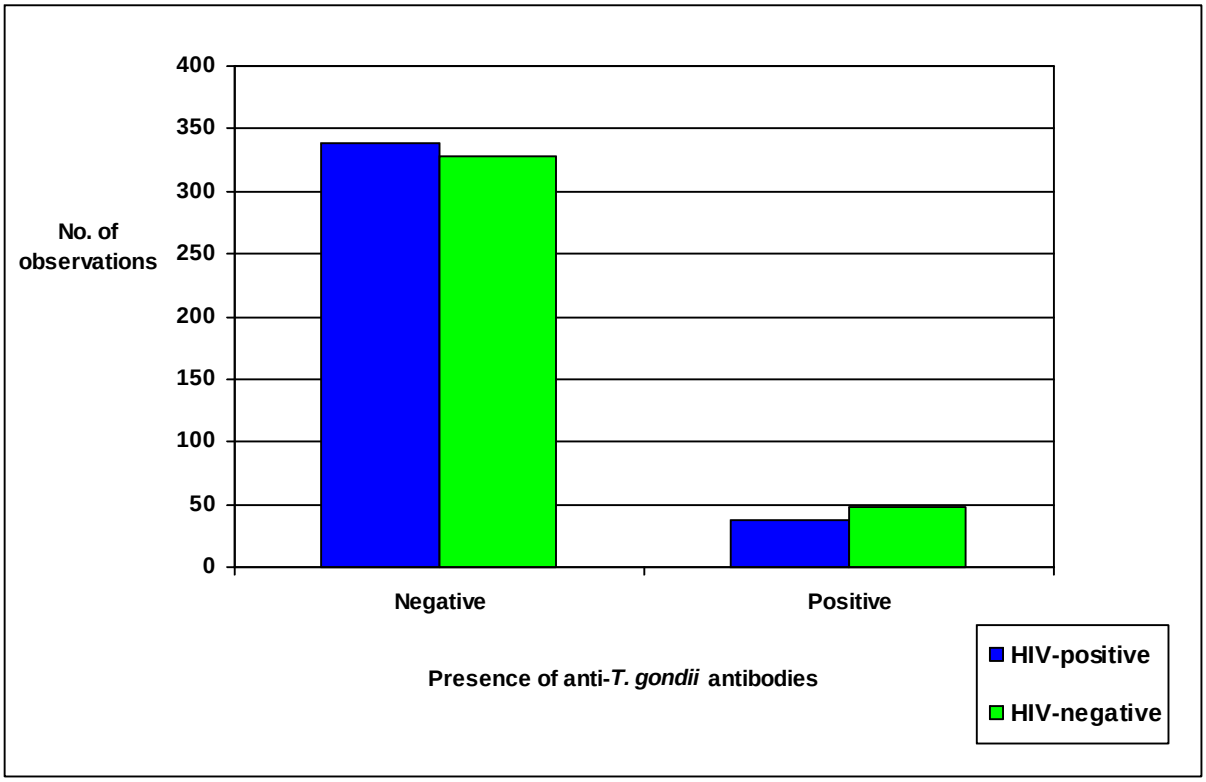


Figure 3.1: Anti-*T. gondii*-antibody presence in HIV-positive patients and HIV-negative controls.

3.2. Retrospective serosurvey

Thirty-two of 497 patients tested by EIA possessed antibodies to *T. gondii*, and the overall prevalence was found to be 6.4%.

3.3. Evaluation of the Pastorex agglutination test

In order to evaluate the Pastorex Toxo latex particle agglutination test (Bio-Rad, France), samples were tested in duplicate on different days, with the operator blinded to corresponding results. Table 3.2 summarizes these results.

Table 3.2: Comparison of results obtained using Pastorex agglutination test

Pastorex (First test)					
		+	Indeterminate	-	Total
Pastorex (Second test)	+	28	7	4	39
	Indeterminate	0	0	2	2
	-	1	3	331	335
	Total	29	10	337	376

Using the above table, the reproducibility, which is a measure of closeness of agreement i.e. the percentage agreement between repeat tests of the same specimen, was calculated to be 95.48% (359/376). The kappa statistic, which compares the agreement against that which might be expected by chance, was calculated to be 0.91(C.I. 0.833-0.988), excluding indeterminate results, showing an almost perfect agreement.

A sequential method of testing was used, whereby all positive and indeterminate samples from the previous test, as well some randomly-selected negative samples were retested, using a reference test comparable to the gold standard assay. These results are summarized in Table 3.3.

Table 3.3: Comparison of results obtained using Pastorex agglutination test and reference test (Biomèrieux Toxo-Screen DA)

Pastorex					
		+	Indeterminate	-	Total
Biomèrieux Toxo-Screen DA	+	27 True +	10	0 False -	37
	-	1 False +	7	23 True -	31
	Total	28	17	23	68

Twenty-seven true positive and 23 true negative results were found. One false positive and no false negative results were found. Statistical analysis results, excluding indeterminate results are summarized in Table 3.4.

Table 3.4: Summary analysis of performance of Pastorex agglutination test compared to reference test (Biomèrieux Toxo-Screen DA)

		Confidence Intervals	
	%	Lower	Upper
Sensitivity	100.00	100.00	100.00
Specificity	95.83	95.75	95.91
Positive predictive value	96.43	96.36	96.50
Negative predictive value	100.00	100.00	100.00
False positive rate	4.17	4.09	4.24
False negative rate	0.00	0.00	0.00

The overall accuracy was 73.53% (50/68). The kappa statistic was calculated, once again excluding indeterminate results, and showed an almost perfect agreement (0.96; C.I. 0.88-1). The McNemar Chi-squared test gave a p-value > 0.05, showing no statistical difference between the tests.

The Pastorex agglutination test was compared to the Axsym Toxo IgG automated EIA, which is the routine test used for *T. gondii* detection in South Africa in the public hospital diagnostic laboratory service (NHLS). The results of this comparison are summarized in Table 3.5 and Table 3.6. Once again, indeterminate results were excluded in all calculations.

Table 3.5: Comparison of results obtained using Pastorex agglutination test and routine diagnostic test (Axsym Toxo IgG automated EIA)

		Pastorex			
		+	Indeterminate	-	Total
Axsym Toxo IgG EIA	+	26 True +	6	0 False -	32
	Indeterminate	0	1	4	5
	-	27 False +	11	422 True -	460
	Total	53	18	426	497

Table 3.6: Summary of statistical analysis of Pastorex agglutination test and routine diagnostic test (Axsym Toxo IgG automated EIA)

		Confidence Intervals	
	%	Lower	Upper
Sensitivity	100.00	100.00	100.00
Specificity	93.99	93.96	94.00
Positive predictive value	49.06	48.92	49.19
Negative predictive value	100.00	100.00	100.00
False positive rate	6.01	5.99	6.03
False negative rate	0.00	0.00	0.00

The overall accuracy was 90.14% (448/497) .The kappa statistic was 0.63 (C.I. 0.51-0.76), indicating a substantial disagreement, and the McNemar Chi-squared test gave a p-value < 0.05, showing a significant statistical difference between the tests.

3.4. Prevalence in rodents

None of the 325 tested rodent samples showed the presence of *T. gondii*-DNA.

Table 3.7: Rodent samples tested for presence of *T. gondii*-DNA

Organ	Number of samples tested	Location	Presence of *T. gondii*- DNA
Spleen	300	Port Elizabeth, Limpopo Province and Durban.	0
Heart	20	Johannesburg	0
Brain	5	Johannesburg	0

PCR methods were validated using known positive and negative controls before use. Electrophoresis gels, as shown in Figure 3.2 indicate that the PCR method amplifying the B1 gene was appropriate. A PCR method was therefore validated that could be used in a diagnostic setting.

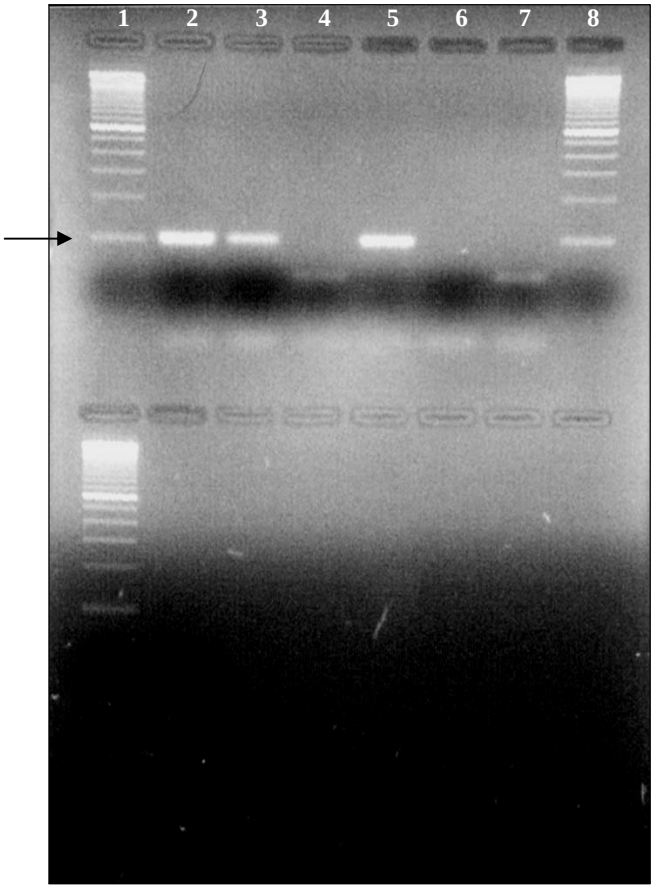


Figure 3.2: Electrophoresis gel of validation PCR, showing amplification of the B1 gene. Upper gel, lanes 1, 8: molecular size marker; lanes 2, 3, 5: known positive controls; lanes 4, 6, 7: known negative controls. Arrow: 100 base pair ladder marker.

Frequently, gels using sample DNA showed atypical bands as illustrated in Figure 3.3.

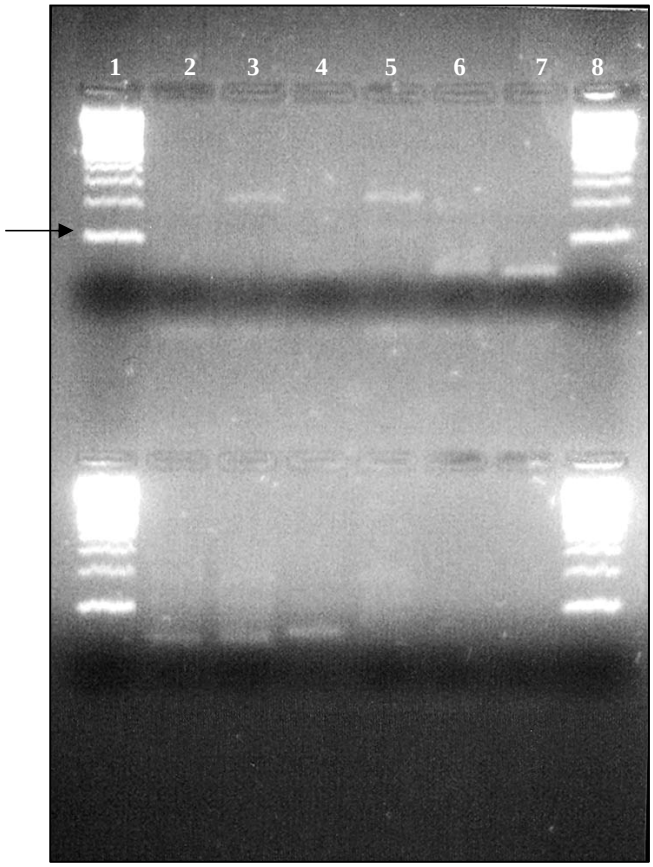


Figure 3.3: Electrophoresis gel of PCR products of the B1 gene. Upper gel, lanes 1, 8: molecular size marker; lanes 2, 4, no amplification; lanes 3,5,6 and 7: atypical bands. Arrow: 100 base pair ladder marker.

The PCR method for strain typing and subsequent enzyme digestion was also validated using known positive and negative controls (Figure 3.4), but no sample DNA could be tested as no positive results from rodent material were obtained.

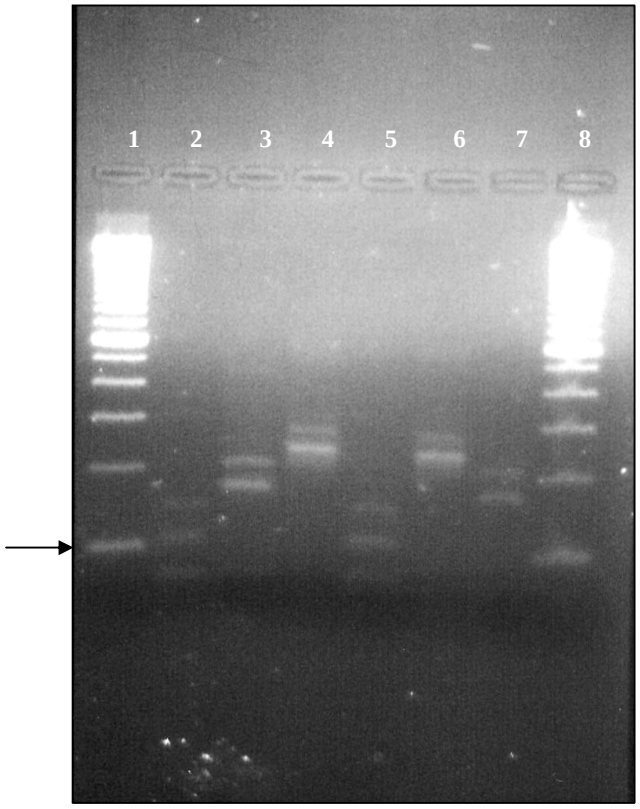


Figure 3.4: Electrophoresis gel showing restriction digests of PCR products of SAG3 gene, demonstrating the 3 *Toxoplasma* strains. Lanes 1, 8: molecular size markers; lanes 2, 5: type I; lanes 3, 7: type II; lanes 4, 6: type III. Arrow: 100 base pair ladder marker.

CHAPTER 4

DISCUSSION

4.1. Introduction

Since its discovery 100 years ago, *T. gondii* has become established as one of the most versatile and successful of all parasites. Its worldwide distribution, broad host range and ability to maintain a benign co-existence with its hosts have enabled its success. The ability of *T. gondii* to be cultured, the fact that it is amenable to genetic manipulation and has excellent animal models, has made studying this organism fairly uncomplicated (Carruthers, 2002).

Although the parasite rarely causes acute disease in healthy individuals, its status as the third most common cause of food-borne deaths in United States and its exceptionally high infection rates show that it is a serious threat to human health (Carruthers, 2002).

4.2. *Toxoplasma gondii* in South Africa

This study has shown the prevalence of anti-*T. gondii* antibodies in an HIV-positive population in Soweto, Johannesburg, to be 9.8%. A comparable HIV-negative control population showed a prevalence of 12.8 %. These prevalences are lower than those reported in a previous study by Jacobs and Mason (1978), which reported a 20% overall prevalence across four sites in South Africa. A more recent study by

Sonnenberg *et al.* (1998) showed a 24.6% prevalence in a cohort of HIV-positive patients originating from rural areas around South Africa. In Johannesburg, a study by Hari *et al.* (2007) showed a similar prevalence to the present report, namely, 8% in HIV-positive individuals at the Helen Joseph Hospital.

The retrospective serosurvey reported here, targeting mainly pregnant women and children, showed a 6.4% prevalence of anti-*T. gondii* antibodies. This prevalence is lower than that reported in a similar study by Schneider *et al.* (1992), which examined the prevalence of *T. gondii* infection in women in KwaZulu-Natal Province and found a 31.3 % prevalence.

These prevalences are also lower than those reported for other parts of Africa as well as other continents. Details of these studies are discussed in Chapter 1.

There are several possible explanations for the low prevalences found in this study, as well as the reported historical differences in prevalences between regions.

In some HIV/AIDS patients, the body may be unable to make sufficient antibodies due to its failing immune system. As a result, antibodies may be undetectable and this may account for the lower prevalence found in the HIV-positive sample. However, evidence against this being an important factor is that a sufficient proportion of HIV-positive patients have detectable levels of antibodies to make such testing of clinical value. All patients participating in this study were enrolled in an antiretroviral (ARV) treatment programme and this may be one of the reasons why those possessing anti-*T. gondii* antibodies did not present with clinical disease. Cotrimoxazole, routinely

used for prevention of *Pneumocystis* pneumonia, also suppresses reactivation of toxoplasmosis. HIV-positive patients admitted to the hospital for treatment were not recruited to this study and there is the possibility that the prevalence in this subpopulation may be higher, and some such patients may be presenting with overt toxoplasmosis.

A less pathogenic clonal lineage of the parasite may be present in South Africa compared to the rest of Africa and other continents. Very little is known genetically about *T. gondii* in Africa. However, a study by Lindström *et al.* (2006), found that the genotype distribution in Uganda appears to be similar to that found in Europe amongst immunocompromised individuals. The type II allele of the SAG2-locus was the most common disease-causing strain found. Nothing is known about *T. gondii* strains affecting humans in South Africa; further studies will be required in this regard.

Previous studies on *T. gondii* in South Africa date back to 1974, which was during the apartheid era and prior to the emergence of the HIV epidemic. Prevalences reported in these earlier studies were higher than those found in this study and other more recent ones. This may be due to differences in socio-economic and cultural factors present nowadays.

During the apartheid era, a large proportion of the black population, in particular, did not have adequate access to good sanitation, clean water, and many grew their own fruit and vegetables as their main source of food. They had more contact with stray animals and rodents, as housing facilities in rural areas were generally very poor.

Many worked on farms where they were at a higher risk of acquiring a *T. gondii* infection due to their close contact with soil and domestic stock reservoirs of toxoplasmosis. Studies on *T. gondii* prevalence during this time therefore found the highest prevalence in the black population (Jacobs and Mason, 1978).

Since the birth of South Africa's democracy in 1994, socio-economic conditions have been changing, with rapid urbanisation. The majority of the population in urban areas has improved access to clean water and sanitation facilities, although breakdowns in these services occur from time to time. Contact with stray cats is limited and due to rodent control campaigns and better living conditions, there might be generally less contact with rodents, although this would be highly variable, as there is still widespread poverty and a wide range of quality of life. Most people buy food from shops and supermarkets and rarely rely on growing their own. There is more education and awareness around good hygiene and washing of fruit and vegetables as well as the importance of properly cooked food.

The development of better farming practices and the widespread use of freezers and frozen meat, as well as pasteurized milk, means that many of the risk factors for acquiring *T. gondii* infection have been reduced.

In the city of Johannesburg, where this study was carried out, there are a large number of apartments and townhouses rather than traditional houses, many of which do not have gardens and do not allow occupants to own pets. As a result, many children growing up in such environments have less contact with soil and cats and therefore

have less chance of acquiring a *T. gondii* infection. Cats are not popular companion animals in most black households, for reasons of superstition.

Climatic conditions may account in some part for the global variation in *T. gondii* prevalence. The climate across much of Africa is generally uniform with considerably less climatic variation between seasons, compared to Europe and North America. *T. gondii* prevalence on this continent is lower than that of Europe and North America; climatic and topographical factors may account for these lower prevalences.

In South Africa, the climate may also contribute to the differences in prevalence between different provinces. In KwaZulu-Natal Province, a study by Bhigjee *et al.* (1999) showed that toxoplasmosis was the most frequent cause of intracranial mass lesions (comprising 52%) in HIV-positive patients. Modi *et al.* (2004), however, found that tuberculosis, not toxoplasmosis, was the most frequent cause of focal brain lesions in a Gauteng population. As discussed in Chapter 1, KwaZulu-Natal and Gauteng Provinces fall in different climatic regions. KwaZulu-Natal is generally hot with high humidity and rainfall while Gauteng generally has low humidity and moderate rainfall. These differences in climate may to contribute to the differences in prevalences. Prevalences might be expected to be higher in hotter, wetter areas as these conditions are more favourable for the sporulation of oocysts. This trend is also evident in the seroprevalence of *T. gondii* in sheep in South Africa, which is higher in the humid costal regions (KwaZulu-Natal - 6.3%, Eastern Cape - 7.75%) and lowest in the arid regions (Gauteng - 6%, Free State - 2.7%) (Samra *et al*., 2007).

Global variation in prevalence may also be attributed to differences in cultural habits. In France, the high *T. gondii* prevalence (up to 75%) has been related to a preference for eating raw or undercooked meat. In Thailand, where Buddhism is the main religion, it is considered a sin to kill any living being. There are therefore large numbers of stray cats which increase the risk of *T. gondii* transmission to humans (Sukthana, 2006). Care of pets is also a factor influencing *T. gondii* prevalence in a population. In Brazil and Mexico, cats are fed leftovers and raw viscera, which have been identified as risk factors for human *T. gondii* infection, and in Thailand, cats are rarely trained to defecate in litter boxes, making the contaminated environment a major risk factor in the human population.

In South Africa, the number of feral cats is relatively low and there may be greater awareness about good hygiene, especially with regard to the care of pets. Raw meat is rarely consumed, and in some religions certain animals are not eaten at all. Hindu and Muslim people, respectively, do not eat beef or pork, and therefore consume relatively more mutton or lamb. As discussed in Chapter 1, sheep are more susceptible to *T. gondii* infection and the handling and consumption of mutton and lamb therefore poses more risk for acquiring infection. This may also account for the higher occurrence of *T. gondii* in KwaZulu-Natal Province where there are a greater number of Hindu and Muslim people.

Differences between reported prevalences may also be due to different sampling procedures and assay methods. Various tests have different sensitivities, specificities, false positive rates and false negative rates. These factors need to be considered when comparing studies. Generally, more sensitive and specific assays are available

nowadays and this could account for the variability in prevalences reported in more recent studies.

4.3. Evaluation of the Pastorex agglutination test

Diagnosis of toxoplasmosis can be made indirectly by serological techniques, or directly by PCR or hybridization, histology or isolation. Direct methods are optimal for a definitive diagnosis, especially in HIV-positive patients, where there is the risk of a reactivated infection. This, however, is not always practical, especially in developing countries such as South Africa, as these direct methods are often expensive, technically demanding, and can be invasive and time-consuming.

Serological methods therefore are still widely used and can be very useful in the right clinical setting. A negative serological result for *T. gondii* is helpful as it almost rules out TE as a diagnosis in HIV/AIDS patients.

The Pastorex agglutination test was therefore evaluated as it is a simple, cheap test, which, if validated, would make screening for toxoplasmosis exposure more accesible for the general population.

The test showed a very high accuracy when first and second round results were compared to one another, and when compared to both the reference (Biomèrieux Toxo-Screen DA) and routine methods (Axsym Toxo IgG (Abbott) automated EIA). This shows that results with the Pastorex test are highly reproducible. No statistical

difference was found between the Pastorex test and the reference method, but there was a statistical difference between the agglutination test and the routine EIA method. Different sets of sera were used for these two comparisons and this may account for the different findings.

The sensitivity of a test is its ability to correctly yield a positive result. The sensitivity of the Pastorex test when compared to both the routine and reference methods was 100%.

The ability of a test to correctly yield negative results is termed the specificity. The specificity of the Pastorex test compared to both the routine and reference methods was 95.83% and 93.99% respectively.

Values for both these parameters were very high for the Pastorex test, indicating that it is both a very sensitive and specific test. The fact that the sensitivity is higher than the specificity means that a lower rate of false negatives than false positives can be anticipated. In the common clinical settings of toxoplasmosis testing (immunosuppression and pregnancy), it is arguably better to pick up some false positives and apply confirmatory testing, rather than to miss any and regard them as negative.

A false positive result occurs when a test falsely identifies a person as having a disease or infection when they in fact do not. False positive rates of 4.17% and 6.01% were found when comparing the test to the reference and routine methods. A false negative result occurs when a test falsely identifies a person as not having a disease or

infection when they in fact do have it. The false negative rate of this test was 0%, indicating that the Pastorex agglutination test has a higher rate of finding patients positive for anti-*T. gondii* antibodies when they do not in fact posses any, but it does not identify patients who do have anti-*T. gondii* antibodies as negative. This indicates that negative results with the Pastorex test are reliable.

Perhaps more important measures of a test are the positive predictive and negative predictive values. The positive predictive value (PPV) is the probability that a person who is identified as positive by the test, does in fact have the disease. The negative predictive value (NPV) is the probability that a person identified as negative by the test truly does not have the disease. For the purposes of a screening test the NPV is of interest. Compared to both routine and reference methods, the NPV was 100%, indicating again that negative results with the Pastorex test are reliable.

Overall the Pastorex agglutination test compared well to the other two methods. The methodology was fairly simple and did not require a high level of expertise. The interpretation of results, however, is subjective and this maybe problematic as indeterminate results may often be found. A further problem is the occurrence of false positive results, as these could have an impact on the treatment of patients.

A very good characteristic of this test is the fact that it does not yield false negative results, indicating that negative results are reliable. This is an important factor in the management of *T. gondii* infections, especially in HIV/AIDS patients.

4.4. *Toxoplasma gondii* in rodents

The role of rodents in the transmission of *T. gondii* has always been considered an important one. In this study the *T. gondii* prevalence in rodents across four sites in South Africa was investigated, but no positive results were obtained. It is unlikely that *T. gondii* is absent in rodents and there are several possible reasons as to why no positive results were obtained.

The testing method used may not have been appropriate or did not function appropriately. In this study, however, PCR diagnostics were used. PCR is considered to be a very sensitive and accurate technique and is widely used for *T. gondii* diagnosis. The method was successfully validated using known positive and negative specimens and each DNA extraction and PCR reaction mix included internal controls ensuring good quality control. One-way traffic-controlled rooms were used in each step of the testing procedure to further ensure no contamination. It is unlikely then that the testing method accounted for the lack of positive results.

The quality of the material used in the testing may not have been optimal. Rodents were trapped by either break-back or Sherman traps (Taylor *et al.*, 2008). The time taken for the rodents to be collected and organs removed and preserved from the time that they were initially trapped is very important. The longer the time taken, the lower the quality of the sample material becomes. There is evidence for this in this study as many electrophoresis gels of PCR products showed atypical bands indicative of poor material quality (Figure 3.4, Chapter 4). There was no way to control for this, as residual material from a previous study was used.

The type of material available was also not optimal. Ideally brain or heart tissue would be best used as there are usually greater numbers of *T. gondii* cysts found here. Only spleen tissue was available for use in this study. The small number of brain and heart tissue samples prospectively collected also did not yield any positive results, but once again the quality of the material received could not be controlled.

There is also the possibility that rodents are no longer as important in the transmission of *T. gondii* to humans as in the past. In principle, they have less contact with humans due to urbanization, rodent control programmes, improving sanitary conditions and increased health awareness. However, suboptimal human living conditions usually favour increased rodent breeding and subsequent increased contact with humans (Taylor *et al.*, 2008). There is intermittent informal evidence (e.g. newspaper reports) that commensal rodents emerge as public health problems from time to time in certain areas in Gauteng Province and elsewhere.

Birds (including chickens), have recently been recognised as an important source of infection. Chickens feed from the ground and therefore presumably have ready contact with *T. gondii* oocysts, and have a greater chance of infecting humans through food preparation and consumption. In rural and peri-urban areas of South Africa where free-ranging chickens are found abundantly, they may have a greater role to play than rodents. Further studies will be done to investigate this possibility.

CHAPTER 5

CONCLUSIONS

The results of this study have shown that *T. gondii* infection is present in South Africa, but its prevalence is much lower than in other parts of the world. This low prevalence means that previously unexposed people are at risk of acquiring an acute infection, which may be passed on congenitally in pregnant women, or which could be life-threatening in HIV/AIDS patients.

Rapid and accurate diagnosis in such patients is therefore essential to ensure effective treatment and prevention of severe disease. This is not an easy task, especially in South African populations where other infections, especially tuberculosis, are very common. Distinguishing a tuberculosis central nervous system infection from toxoplasmosis is a common diagnostic challenge and in populations such as this where tuberculosis is highly endemic, patients are probably often not appropriately treated for toxoplasmosis.

It is recommended that the Pastorex Toxo latex particle agglutination test be used as a screening test as it is cheap, quick and reliable to rule out toxoplasmosis as a diagnosis if negative. All positive and indeterminate results should be retested using another more specific test or by direct methods such as PCR. Care should also be taken when interpreting the results of tests in severely immunocompromised individuals, as antibodies may be undetectable in some who do in fact have an infection. Direct methods are preferable in such situations.

From the public health perspective, in a general population with such a low prevalence and few risk factors for transmission of *T. gondii*, strict control measures and screening programmes would be expensive and unnecessary. However, interventions could benefit certain subpopulations at particular risk, as discussed below.

Newly-diagnosed HIV-positive, or pregnant, patients should be screened for previous *T. gondii* exposure so that adequate precautions can be taken by both the clinician and the patient to prevent an acute or reactivated infection. Continued awareness around good hygiene and infection control should be emphasised by clinicians and public health professionals, especially with respect to pregnant women and HIV-positive individuals.

This study helped to answer questions relating to the seroprevalence and diagnosis of *T. gondii* in South Africa. Many questions still remain to be answered, however, to fully understand the impact of this parasite in our country and to help to implement solutions aimed at reducing risk for this curable but potentially fatal disease. The growing AIDS epidemic and the recent detection of drug-resistant HIV strains is a disturbing reminder that opportunistic infections such as toxoplasmosis, even in low prevalences, remain a major potential threat to human health.

APPENDIX A

Patient information and consent forms

Title of Project:

Studies of *Bartonella* and *Toxoplasma gondii* species in human populations in South Africa

Patient information sheet

Hello. My name is _____ and I would like to invite you to participate in a study. Your participation in this study is completely voluntary. If you decide not to participate, it will not affect your treatment in any way and you may also change your mind at any time. As we discuss the information, please feel free to ask me any questions.

I am studying infections called 'toxoplasmosis' and 'bartonellosis', caused by germs most commonly transmitted from humans animals and some foods. These infections can sometimes cause illness in humans. I would like to test your blood for signs of these infections, and ask your permission to take, at the same time that blood is taken from a vein in your arm for other tests routinely done in this clinic, two extra 5ml (2 teaspoons) tube of blood. This blood will be used to test for the presence of these infections and will not be used for any other purpose. Your blood will be disposed of safely once we have carried out these tests. Only the people involved in this study will have access to your blood and to these results. Your doctor will be given the results of my test, and will tell you about them when you return to the clinic for your normal appointment.

Thank you for your time. Once you have asked me any questions, you can decide in your own time whether you want to give the blood sample or not.

If you would like any more information on the study, you can contact any of the researchers that are here, or Miss K. Kistiah (011) 555 0332 or Miss A.N. Trataris (011) 555 0331





NICD

Title of Project:

Studies of *Bartonella* and *Toxoplasma gondii* species in human populations in South Africa

Informed consent form*:*

I have understood the information (verbal and/or printed) about this study, and agree to donate a sample of blood to be tested only for toxoplasmosis and bartonellosis , and I understand that any remaining blood will be destroyed and not used for any other purpose.

I understand that I have been invited to participate in this study, and I understand that my agreeing to participate is fully voluntary and I can withdraw at any time.

Subjects name:

Signature

Date:

Consent record for blood tests for toxoplasmosis and bartonellosis.

Age: Gender:	Date:
Information provided and written consent witnessed by (print):	Sign:
Blood sample taken by (print):	Sign:
Study sample number:	

Ethical Clearance Certificates

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Kistiah et al

CLEARANCE CERTIFICATE	**PROTOCOL NUMBER M070637**
PROJECT	Studies of Bartonella and Toxoplasma gondii Species in Human Populations in South Africa
INVESTIGATORS	Miss K Kistiah et al
DEPARTMENT	Virology & Communicable Dis.
DATE CONSIDERED	07.06.29
DECISION OF THE COMMITTEE*	APPROVED UNCONDITIONALLY

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 07.08.06 **CHAIRPERSON** .....
(Professors PE Cleaton-Jones, A Dhai, M Vorster, C Feldman, A Woodiwiss)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Dr J Frean

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10005, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Kistiah

CLEARANCE CERTIFICATE	**PROTOCOL NUMBER M070347**
PROJECT	Epidemiology of Toxoplasmosis in South Africa
INVESTIGATORS	Miss K Kistiah
DEPARTMENT	School of Pathology
DATE CONSIDERED	07.03.30
DECISION OF THE COMMITTEE*	APPROVED UNCONDITIONALLY

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 07.08.06 **CHAIRPERSON** .....
(Professors PE Cleaton-Jones, A Dhai, M Vorster, C Feldman, A Woodiwiss)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Dr AJ Frean

DECLARATION OF INVESTIGATOR(S)

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PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

AESC 3

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2008/50/01

APPLICANT: Ms K Kistiah

SCHOOL: School of Pathology -- Virology and Communicable disease
DEPARTMENT:
LOCATION:

PROJECT TITLE: **Epidemiology of toxoplasmosis in South Africa**

Number and Species

400 Rattus spp

Approval was given for the use of animals for the project described above at an AESC meeting held on 28.10.2008. This approval remains valid until 28.10.2010

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and to the following additional conditions:

Liaise with Ms A.N Tratatis on the recollection of samples of tissue, CO_2 immersion and not ether

Signed: _____ Date: 31/10/2008
(Chairperson, AESC)

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ Date: 31/10/2008
(Registered Veterinarian)

cc: Supervisor:
Director: CAS

Works 2000/lain0015/AESCCert.wps

APPENDIX B

IFL protocol

Washed *Toxoplasma* tachyzoites are distributed on 8-well Teflon-coated microscope slides.

Slides are left to air dry

Slides then fixed in cold acetone for 20 minutes and stored frozen until used

1. Make serum dilutions of 1/10, 1/30,1/90,1/270… in PBS
2. Rinse slides in PBS and dry between wells
3. Add 15µl of each dilution to each well, starting with the lowest dilution
4. Incubate for 30 minutes at room temperature
5. Wash 3 times in PBS for 5 minutes at room temperature
6. Dilute FITC conjugate in Evans Blue (1/10 000) mixture according to how much is needed
7. Add 15µl of this mixture to each well
8. Incubate for 30 minutes at room temperature
9. Wash 3 times in PBS for 5 minutes at room temperature
10. Mount in glycerol medium (non-fading Vectashield, Vector Laboratories Inc., Burlingame, CA", or "3µm)
11. View under fluorescent microscope equipped with filters for fluorescein isothiocyanate (FITC)

QIAgen tissue protocol,

(QIAamp DNA mini kit)

1. Cut up to 25 mg of tissue (up to 10 mg spleen) into small pieces, place in a 1.5 ml microcentrifuge tube, and add 180 µl of Buffer ATL

2. Add 20 µl Proteinase K, mix by vortexing, and incubate at 56°C until the tissue is completely lysed. Vortex occasionally during incubation to disperse the sample

3. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid

4. Set up a 200 µl control

4. Add 200 µl Buffer AL to the sample, mix by pulse-vortexing for 15 s, and incubate at 70°C for 10 min. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid

5. Add 200 µl ethanol (96-100%) to the sample, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid

6. Carefully apply the mixture from step 4 (including the precipitate) to QIAamp spin column (in a 2 ml collection tube) without wetting the rim. Close

the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate

7. Carefully open the QIAamp spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate

8. Carefully open the QIAamp spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min

9. Place the QIAamp spin column in a new 2 ml collection tube (not provided, use 1.5 ml tube with lid removed) and discard the collection tube containing the filtrate. Centrifuge at full speed for 1 min

10. Place the QIAamp spin column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp spin column and add 200 µl Buffer AE or distilled wafer. Incubate at room temperature for 5 minutes and then centrifuge at 6000 x g (8000 rpm) for 1 min

11. Repeat step 10

Burg *et al.* (1989) PCR protocol: *J. Clin. Microbiol.*1989; 1989; 27: 1787-1792

Nested PCR; 97-bp fragment of B1 gene

In clean room make master mix:

1. Thaw PCR reagents (keep dNTP mix, Polymerase and water on ice)
2. Vortex all PCR reagents before pipetting
3. Add PCR mix reagents from largest volume to smallest into a PCR tube

1° PCR per tube:

20.85 µl Water

5µl of 10x buffer

3µl of 25 mmol/L $MgCl_2$

0.5µl of 10mmol/L dNTP's

0.2 µl of 100 µmol/L primer B1

0.2 µl of 100 µmol/L primer B2

0.25µl (5U/µl) AmpliTaq Gold DNA Polymerase

2° PCR per tube:

35.85 µl Water

5µl of 10x buffer

3µl of 25 mmol/L $MgCl_2$

0.5µl of 10mmol/L dNTP's

0.2 µl of 100 µmol/L primer B3

0.2 µl of 100 µmol/L primer B4

0.25µl (5U/µl) AmpliTaq Gold DNA Polymerase

* Vortex PCR mix before adding Polymerase *

4. Aliquot 30µl(for 1°) and 45 µl (for 2°) into PCR tubes
5. Tubes can be stored frozen

Primary PCR (50µl volume): uses primers B1 and B2

1. Thaw, vortex and quick spin DNA template

2. Thaw and label PCR tubes according to protocol

3. Use filter tips to add 20 µl DNA template in DNA hood .Keep tubes closed in between samples to limit contamination

4. For PCR control, add 20µl water

5. Vortex and quick spin samples

6. Place in PCR machine
   Cycling Parameters: 94°C for 12 minutes (to activate enzyme)

   - 35cycles of: 94°C for 15s, 50°C for 30s, 72°C for 45s

   - final extension at 72°C for 5 minutes

7. Store 1° PCR templates in the fridge until ready to perform 2°PCR. For long term storage place them in a box in the freezer

Secondary PCR (50µl volume): uses primers B3 and B4

1. Label 2° PCR tubes according to protocol

2. Transport to the nested PCR area

3. Vortex and quick spin 1° PCR template

4. Make a 1/20 dilution of the 1° PCR product by adding 5µl of 1° PCR template to 95 µl Milli-Q water. Keep tubes closed in between samples to limit contamination

5. Vortex, then add 5 µl of this dilution to the 2° PCR master mix

8. Vortex and quick spin samples

6. Place in PCR machine
   Cycling Parameters: 94°C for 12 minutes (to activate enzyme)

   - 35cycles of: 94°C for 15s, 50°C for 30s, 72°C for 45s

- final extension at 72°C for 5 minutes

7. 2° PCR templates can be left in the PCR machine overnight at 4°C, refrigerated or frozen until ready for electrophoresis

dNTP mix: 10mmol working dilution :

100 µl of each nucleotide

600 µl Milli-Q water

Total volume is 1ml

Primers :

Primers are reconstituted according to supplier's specifications (Thermo Fisher Scientific,Germany) by adding Milli-Q water in the following volumes:

Toxoplasma B1 : 446,2 µl
(5'- GGA ACT GCA TCC GTT CAT GAG -3')

Toxoplasma B2 : 271,0 µl
(5'- TCT TTA AAG CGT TCG TGG TC -3')

Toxoplasma B3 : 255,1 µl
(5'- TGC ATA GGT TGC AGT CAC TG -3')

Toxoplasma B4 : 470,7 µl
(5'- GGC GAC CAA TCT GCG AAT ACA CC-3')

Gel electrophoresis

Method:

1. Wipe gel chamber with water and dry
2. Prepare the small gel-setting chamber by adding combs
3. Measure 0, 6 g of agar in a 100ml flask, add 30 ml TBE buffer and mark level
4. Dissolve over a Bunsen burner or in a microwave; boil twice and leave to cool for a few minutes (60°C).Add de-ionised water if any has evaporated
5. Once cool, add one drop of ethidium bromide and swirl to mix
6. Pour the gel into the chamber and leave to set
7. Remove combs, place in tank and pour 1X TBE buffer over
8. Pipette 2µl of loading buffer (Blue Juice) onto parafilm for each PCR sample
9. Add 10µl of PCR product to each drop, pipette to mix and load into wells
10. Load 3µl of molecular size marker into the first and last well
11. Attach leads; run from red (in front) to black at 95 volts for 45 mins

Agarose gel; 2% of 30 ml volume

Agarose	0.6g
TBE buffer (1X)	30ml
Ethidium bromide (10mg/ml)	one drop

Molecular size marker

20µl Step Ladder

40µl loading buffer

40µl Milli-Q water

This marker produces a ladder of bands of between 100 and 1500 base pairs, namely 97 BP (B1 PCR)

331 BP (SAG3 Primary PCR)

226 BP (SAG3 Secondary PCR)

TBE Electrophoresis Buffer (working solution); 1 litre

TBE (10X stock)	100ml
Deionised water	900ml

Grigg *et al*. (2001) PCR protocol: *J .Infectious Diseases.* 2001; 184: 633-639

Nested PCR; Surface Antigen Gene 3 (SAG3)

In clean room make master mix:

6. Thaw PCR reagents (keep dNTP mix, Polymerase and water on ice)
7. Vortex all PCR reagents before pipetting
8. Add PCR mix reagents from largest volume to smallest into a PCR tube

1° PCR per tube:

37.2 µl Water
5µl of 10x buffer
3µl of 25 mM $MgCl_2$
1 µl of 10 µM primer 1
1 µl of 10 µM primer 2
0.5µl of 10mM dNTP's
0.3µl AmpliTaq Gold DNA Polymerase

2° PCR per tube:

38.2 µl Water
5µl of 10x buffer
3µl of 25 mM $MgCl_2$
1 µl of 10 µM primer 3
1 µl of 10 µM primer 4
0.5µl of 10mM dNTP's
0.3µl AmpliTaq Gold DNA Polymerase

* Vortex PCR mix before adding Polymerase *

9. Aliquot 48µl(for 1°) and 49 µl (for 2°) into PCR tubes

10. Tubes can be stored frozen

Primary PCR (50µl volume): uses primers 1 and 2, 311bp fragment.

9. Thaw, vortex and quick spin DNA template

10. Thaw and label PCR tubes according to protocol

11. Use filter tips to add 2 µl DNA template in DNA hood. Keep tubes closed in between samples to limit contamination

12. For PCR control, add 2µl water

13. Vortex and quick spin samples

14. Place in PCR machine
    Cycling Parameters: - 35cycles of: 94°C for 30s, 60°C for 30s, 72°C for 30s

15. Store 1° PCR templates in the fridge until ready to perform 2°PCR. For long term storage place them in a box in the freezer

Secondary PCR (50µl volume): uses primers 3 and 4, 226 bp fragment.

8. Label 2° PCR tubes according to protocol

9. Transport to the nested PCR area

10. Vortex and quick spin 1° PCR template.

11. Add 1 µl of 1° PCR template to 2° PCR master mix

12. Vortex and quick spin samples

13. Place in PCR machine

14. Cycling Parameters: - 35cycles of: 94°C for 30s, 60°C for 30s, 72°C for 30s

15. 2° PCR templates can be left in the PCR machine overnight at 4°C, refrigerated or frozen until ready for electrophoresis

dNTP mix: 10mmol working dilution :

100 µl of each nucleotide

600 µl Milli-Q water

Total volume is 1ml

Primers :

Primers are reconstituted according to suppliers specifications (Thermo Fisher Electron, Germany) by adding Milli-Q water in the following volumes to obtain a 100pmol/ µl concentration:

Primer 1 (P43AS1-Fext) : 540,2 µl
(5'- CAA CTC TCA CCA TTC CAC CC -3')

Primer 2 (P43AS1-Rext) : 343,5 µl
(5'- GCG CGT TGT TAG ACA AGA CA -3')

Primer 3 (P43S2-Fint): 480, 0 µl
(5'- TCT TGT CGG GTG TTC ACT CA-3')

Primer 4 (P43AS2) : 434,6 µl
(5'- CAC AAG GAG ACC GAG AAG GA-3')

Primers are further diluted 1/10 to obtain a 10pmol/ µl working solution

Enzyme Digestion

Grigg *et al.* (2001)

Restriction enzyme: NciI

1. Mix :

   7.5 µl MilliQ water

   2.0 µl Buffer (TANGO X10)

   0.5 µl NciI enzyme

   10 µl PCR product

2. Incubate at 37°C for 3 hours
3. View using gel electrophoresis

Restriction enzyme: BcnI

1. Mix :

   7.0 µl MilliQ water

   2.0 µl Buffer (TANGO X10)

   1.0 µl BcnI enzyme

   10 µl PCR product

2. Incubate at 37°C for 3 hours
3. View using gel electrophoresis
4. Restriction enzymes cut type I strain – twice (154 BP)

   type II strain- not cut (298 BP)

   type III strain -once (220 BP)

Toxoplasma gondii cultivation

Human Foreskin Fibroblasts (HFF) Cells:

1. Remove geneticin medium from flask of confluent HFF cells
2. Wash with 5ml sterile PBS
3. Incubate with 1ml of 0,125 % trypsin diluted in PBS for 10 minutes at 37°C
4. Add 1ml of Gentamicin medium and spilt cells into 4 new flasks (0,5 ml per flask)
5. Add 5ml Gentamicin medium to each of 3 flasks, and 5ml Geneticin medium to the fourth flask, to maintain stock culture
6. Incubate at 37°C, 5% CO2 incubator until a confluent monolayer of cells grow (+/- 1 week)

Toxoplasma gondii:

1. Select a confluent HFF cells flask, change medium
2. Select a freshly lysed HFF/Toxoplasma flask. Remove cells from the walls with a pipette, transfer desired amount of Toxoplasma to the new HFF flask

   2 day passage: 6-10 drops

   1 week passage: 50 µl

   Overnight passage: 1-1, 5 ml
3. Recap flasks loosely and place in incubator

Collection of parasites:

1. Remove cells from walls with a pipette
2. Using a needle and syringe, remove parasites and medium
3. Remove needle and attach syringe to 3µM Isopore filters, Millipore, Billerica, MA, USA
4. Press down gently to allow all liquid to pass through
5. Centrifuge at 2 500 rpm for 5 minutes
6. Discard supernatant
7. Disturb sediment slightly, wash with 5ml PBS (2 500 rpm for 5 mins)
8. Remove PBS and collect sediment
9. Can be frozen as a pellet at -20°C

Medium:

Prepare in sterile Falcon tube:

DMEM	44ml
Hepes (1M)	0.5 ml
FCS	5ml
L-Glutamine (200 Mm)	0.5ml
Gentamicin (10mg/ml)	0.1ml

For stock culture of HFF add Geneticin (0.4ml /50ml of stock 50 mg/ml) instead of Gentamicin.

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