Aspects of Infectious Retinitis in Immunocompetent and HIV infected people

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Abstract

Various issues associated with infectious retinitis in both the immunocompetent and immunocompromised patient are examined.

With the introduction of Highly Active Anti-retroviral therapy (HAART), the clinical manifestations of AIDS are changing. Patient survival is increasing with the lowering of HIV antigenaemia, and the recovery of CD4+ cell numbers. It remains unknown, however, whether the numerical increase is paralleled by a concomitant recovery in immune function. An investigation was undertaken into the survival of patients with CMV retinitis both before and after the introduction of HAART.

The clinical appearance of retinitis associated with various aetiological agents is changing and is less well characterised than before. The role of molecular biology is therefore becoming increasingly important as a diagnostic aid, and in this context, the role of the polymerase chain reaction (PCR) in the diagnosis of Toxoplasma (T.gondii) retinochoroiditis by the amplification of DNA from various ocular samples has been investigated.

Given that most HIV/AIDS is in the developing world, ocular aspects of AIDS both in the profoundly immunosuppressed and in those patients who have not long seroconverted are described in a population cohort from a rural population in Uganda.
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Punctate inner retinal lesions:

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Publications and Presentations arising from this work


**Paper in Preparation**

1) Can *T. gondii* DNA be detected in aqueous samples from patients with active retinochoroiditis and anterior uveitis by PCR?

**Presentations**

1) Use of Intravitreal therapy in the management of CMV retinitis in eyes that have undergone pars plana vitrectomy with silicone oil insertion. Free paper at the fourth International Ocular Inflammation Society meeting Sept 1996

2) Rapid detection of *Toxoplasma gondii* DNA in small ocular samples. Free paper at the Annual Congress in Birmingham 1997.

2) Rapid detection of *Toxoplasma gondii* DNA in small ocular samples. Poster; Association for Research in Vision and Ophthalmology Florida; May 1997

3) Increasing survival in AIDS patients with cytomegalovirus retinitis treated with HIV protease inhibitors. Walsh J C, Jones C D, Gazzard B G, Mitchell S M.

Key to abbreviations

ARC = AIDS related complex
AIDS = Acquired immune deficiency syndrome
ARN = Acute retinal necrosis
AZT = Zidovudine
°C = Degrees Celsius
CI = Confidence Interval
CMV = Cytomegalovirus
CMVR = Cytomegalovirus retinitis
CSF = Cerebrospinal fluid
ddi = Didanosine
DNA = Deoxyribonucleic acid
dNTP = Deoxynucleotide triphosphate
ELISA = Enzyme-linked Immunosorbent Assay
ESA = Excreted/secreted antigens
FDC = Follicular dendritic cells
Fg = Femtogram
G = Gauge
HAART = Highly Active Anti-retroviral therapy
HCl = Hydrochloride
HHV-8 = Human Herpes virus type 8
HIV = Human Immunodeficiency virus
HIV-1 = Human Immunodeficiency virus, Type 1
HPV = Human Papilloma Virus
HTLV-1=Human T-lymphocyte virus, Type 1

IFN γ=Interferon γ

Ig (G, M, A, or E)=Immunoglobulin class (G, M, A, or E)

I.V.=Intravenous

Kb=Kilobase

KCl=Potassium chloride

KS=Kaposi’s sarcoma

M=Molar

Mg²⁺=Magnesium

MgCl₂=Magnesium chloride

µg=Microgram

µl=Microlitres

µm=Micrometres

µM=Micromolar

mM=Micromolar

ml=Millilitres

mm=Millimetre

n=number

NaCl=Sodium chloride

Ng=nanogram

NHC=Natural history cohort

NHL=Non-Hodgkin’s lymphoma

NK=Natural killer T-cells

nm=nanometres
NRTI=Nucleoside reverse transcriptase inhibitors
OD=Right eye
OH=Hydroxyl
OS=Left eye
p=Probability
PCP=Pneumocystis carinii pneumonia
PCR=Polymerase chain reaction
PI=Proteinase inhibitor
PORN=Punctate outer retinal necrosis
PY=Person years
RD=Retinal detachment
rpm=revolutions per minute
RRD=Relative risk of Death
SD=Standard deviation
SDS=Sodium Dodecyl Sulphate
Taq=Thermostable DNA polymerase derived from the bacterium T.aquaticus
TBE=Tris Borate EDTA
TE=Tris EDTA buffer
TNF=Tumour necrosis factor
U=unit
U.K.=United Kingdom
U.S.A.=United States of America
UV=Ultraviolet
$\chi^2$=Chi-squared test
Chapter 1 : Introduction

Summary of Thesis

Various aspects of ocular disease, particularly related to retinitis, in both the immunocompetent and immunosuppressed patient are investigated herein. The diagnosis and management of retinitis frequently presents the ophthalmologist with a difficult clinical challenge; ocular disease caused by a specific pathogen may have various manifestations, and whilst the appearance of the lesions caused by different infectious organisms may appear similar, both the natural history and treatment of the disease process may be very different. Accurate and quick diagnosis is often vital in order to minimize the amount of associated retinal damage, to try and reduce further unwanted sequelae, such as the development of rhegmatogenous retinal detachment, and to ensure timely therapeutic intervention. Commonly drugs with potentially dangerous side effects are used, and if used inappropriately may lead to a deterioration of the patient’s clinical state. An obvious example of this is the inappropriate use of steroids.

Chapter 1 of this thesis gives a background summary of the subjects examined. The thesis is then divided broadly into three further chapters detailing the work undertaken. A final chapter presents an overall summary of results and conclusions.

Chapter 2 is concerned with the diagnosis of Toxoplasma retinochoroiditis. Currently this diagnosis remains essentially a clinical one, as current laboratory investigations are limited in the diagnosis of this condition. Toxoplasma serology, although specific for the organism, is an insensitive tool for the diagnosis of ocular disease as a high percentage of the population have antibodies to the parasite without any evidence of ocular involvement and antibody titres may remain elevated for
long periods of time. Furthermore there appears to be no relationship between Dye test titres and ocular disease activity. The investigation of local antibody production has been found to have variable results. Under such circumstances molecular biological tools can be invaluable as a diagnostic aid.

The main laboratory part of this work has been concerned with the investigation of the amplification of target sequences of DNA from within three different *T. gondii* genes by the polymerase chain reaction. The hypothesis that it is possible to detect *T. gondii* DNA from aqueous samples from patients with active retinochoroiditis was examined. Reactions were optimized for sensitivity using known quantities of *T. gondii* DNA that had been spiked into distilled water. These reactions were then repeated using known quantities of *T. gondii* DNA that had been spiked into aqueous samples. From this work it was concluded that primers directed against target sequences from within the B1 gene were the most sensitive. These primer sequences were tested against available data on Genbank and were further tested against DNA from a variety of common pathogens, and against human DNA to ensure specificity. PCR products were sequenced to confirm they were the expected oligonucleotides. Finally aqueous and vitreous samples from patients with active retinochoroiditis and paraffinised retinal sections containing tissue cysts were subjected to these PCR protocols.

**Chapter 3** of this thesis presents a study into the survival of patients after the diagnosis of CMV retinitis. Since the advent of HAART therapy the clinical characteristics of cytomegalovirus retinitis have changed. When this condition was originally described in AIDS patients it was universally a pre-morbid diagnosis, however since the early 1980’s there has been a gradual improvement in life expectancy in these patients. A cohort of CMV retinitis patients which has been
followed prospectively since October 1992 was reviewed to determine whether the use of combination antiretroviral therapy has been associated with increased survival in this group of patients. This work demonstrates that it is the use of Proteinase Inhibitors (PI's), that has had the single most profound effect on patient survival.

**Chapter 4** presents work that was conducted in rural Uganda. Ophthalmic examination of a Natural History Cohort (NHC) of HIV positive patients and negative controls was carried out. As the examining ophthalmologist I was unaware of the participants HIV status. The study addressed the burden of eye disease caused by HIV-1 in the community incorporating all stages of the diseases.

**Chapter 5** is a summary of the results and conclusions drawn from this work.
Toxoplasma gondii

Background

*Toxoplasma gondii* is an obligate intracellular protozoan parasite and is responsible for the disease toxoplasmosis. It is a well recognized cause of blindness, mental retardation and occasionally the death of congenitally infected infants [1]. The acute acquired disease in adults is only symptomatic in 10%-20% of cases [2]. In this group of patients toxoplasmosis most commonly presents with cervical lymphadenopathy [3]. The disease is also recognized in patients who are immunocompromised as a result of drug therapy [4] or neoplastic disease [5] and more recently in AIDS patients [6]. In AIDS patients toxoplasmosis is emerging as an important cause of infectious retinitis [7].

Epidemiology and Geographic Distribution

*T.gondii* is found in herbivorous, carnivorous and omnivorous animals, including all mammals throughout the world. The high prevalence of the organism in meat is due to the fact that the oocysts have been found in the faeces of approximately 1% of cats. In general, the prevalence of toxoplasma antibodies is less in cold regions, hot and arid areas and at higher altitudes [8].

Prevalence

Toxoplasmosis is one of the most common infections of humans the world over [9], with approximately 500 million people throughout the world having antibodies to *Toxoplasma* [10]. Infection with *Toxoplasma gondii* exists in the chronic asymptomatic form in approximately 50% of the population in the USA, with over 66 million people infected by this protozoan [9]. *T.gondii* is clinically important in the adult for three major reasons; it may cause lymphadenopathy; as an opportunist
it may cause a lethal infection in the immunocompromised, and if it infects
seronegative women during pregnancy it may cause congenital infection of the

It is the most common cause of retinochoroiditis worldwide in humans [12], with
figures ranging from 28% to 55% of all cases of posterior uveitis [13-15].

Transmission

*T.gondii* has a complex life cycle [16]. The principal forms by which it is transmitted
are the tissue cyst (containing bradyzoites) and the oocyst (containing sporozoites)
[17]. Infection with *Toxoplasma* is widespread in animals and man, however,
toxoplasmosis, the disease, is relatively rare. The infection is a zoonosis, and
members of the cat family appear to be the definitive host. *Toxoplasma* infection
occurs worldwide wherever cats are present. Because illness is relatively rare, the
presence of antibodies to *T. gondii* is taken as evidence of infection. Three patterns of
transmission are recognised, adult, childhood and transplacental [18].

Adult transmission

Adult infection starts in late adolescence and increases gradually throughout
adulthood. Infection is primarily by the oral route through ingestion of undercooked
meat or other foodstuffs [19] that contains tissue cysts [16, 20, 21], or of water or food
stuffs contaminated with oocysts [16, 22]. Other forms of transmission include blood
transfusion from asymptomatic people with parasitaemia [23], and bone marrow
transplantation [24], ingestion of raw goat’s milk [19] and from laboratory accidents
[25]. Contamination following contact with the infected saliva and nasal secretions
from a cow with acute toxoplasmosis has been reported [26]. In many areas of the
world the mode of transmission is mixed.
**Childhood transmission**

Childhood transmission starts at about one year of age and 50-70% of children may develop antibodies by the time that they become adolescents. Frequently thereafter seroconversion decelerates and reaches a level state. This is common in Central America and in many moist and tropical countries [27]. It correlates with children playing on the ground in sand and soil, and with soil contamination with cat faeces, especially around the house [22].

**Congenital Infection**

Transplacental transmission was the first recognised means of transmission of *Toxoplasma* to humans [28]. This mode of transmission occurs when the infection is acquired during pregnancy. The incidence of congenital infection varies with the trimester during which the mother becomes infected: the lowest incidence is in the first trimester, and the highest incidence in the third trimester [29]. The more severe sequelae are related to infection acquired from the second to the sixth month of pregnancy: transmission during the third trimester is usually associated with subclinical disease [1]. Some of the factors that determine whether transmission to the foetus will occur are the immunocompetence of the mother, the virulence of the infecting strain of *T.gondii*, integrity of the placenta, and the relationship between time of maternal infection and transplacental passage of antibodies to the foetus [9].

Figures for the rate of foetal infection vary, but in about 33 to 40 percent of cases where a mother becomes infected with *Toxoplasma* will it be transmitted to the foetus [29-31].

It has been estimated that 23% to 38% of babies born with the infection will die or have severe or moderate CNS and ocular damage [32]. The remaining babies with asymptomatic infection may develop active retinochoroiditis, mental retardation, or
seizure disorders as children or young adults. In areas where the incidence rate is higher in childhood and adolescence, most women are immune once they reach their reproductive years.

**Strains of T. gondii**

Evidence that there is more than one strain of *T. gondii* is based on differences in virulence of different strains for laboratory animals, these differences correlate with generation time in tissue culture. Furthermore, an increase in the virulence of a given strain of toxoplasma has also been observed after repeated passages in mice. The virulence of the organism has been found to play an important role in the degree of cell destruction and the time of cyst formation. The less virulent strains producing less destruction in most cell lines, producing tissue cysts earlier. By far the most virulent strain is the RH strain, a cell line that was originally isolated from a human encephalopathy sample. This strain has been found to be invasive for all cell lines, often producing extensive tissue destruction. Strain specific antigenic variability has been demonstrated in *T. gondii*, and amongst commonly shared antigens between different strains, such as the P30 antigen, there is quantitative and probably qualitative differences in the recognition of these antigens as detected by Western blotting. Monoclonal anti-P30 antibody raised against the RH strain has been found to be highly lethal to the RH strain and significantly less so against other strains studied.

**Life Cycle**

Toxoplasma exists in three forms:

1. Oocyst, found only in the cat family and, except for transmission, does not have a role in human infection.
2. Tissue cyst (containing the bradyzoite) that develops in host tissues from trophozoites. The bradyzoite is responsible for the persistence of latent infection in multiple organs.

3. Trophozoite (tachyzoite). This is the invasive form, responsible for the manifestations of acute infection.

**Oocysts**

In the cat, *T. gondii* has both the enteroepithelial and an extraintestinal cycle, whilst in incidental hosts such as humans, it exists only in the extraintestinal cycle. A sexual cycle in the intestines of cats, results in a shedding of oocysts in cat faeces [16].

Following ingestion by the cat of tissues containing the cyst form, bradyzoites are released from disrupted cysts, which then invade the feline enterocytes, differentiating into male and female gametes, which fuse forming a zygote. Within a protective wall the zygote is termed an oocyst, many millions of which are then excreted into the environment in the cat faeces [41]. The potential importance of ingestion of contaminated food and water containing oocysts as a route of infection has been confirmed by Kasper and Ware [42]. They demonstrated the presence of antibodies to stage specific oocyst/sporozoite antigens in the acute and convalescent sera of individuals infected in an outbreak associated with contaminated water.

**Tissue Cysts (Bradyzoites)**

Tissue cysts vary in size from 10–200 μm and contain up to 300 organisms. They are formed within host cell vacuoles with a surrounding membrane elaborated by the organism [43]. This cyst wall and the intracellular environment afford protection against the host defenses. Bradyzoites within the tissue cysts form the principal form
of the organism that persist in chronic (latent) infection. The bradyzoite is the only stage that can initiate the sexual phase. The mechanisms involved in transformation of the tachyzoite to the bradyzoite remain unknown. Bradyzoites do not express detectable levels of the major tachyzoite membrane protein, P30 \(^{17}\).

**Tachyzoites**

The asexual invasive form, measuring 7x3\(\mu\)m \(^{17}\). Although eukaryotes, an intracellular environment is required for survival and multiplication which occurs by endodyogeny \(^{44}\), a process by which two daughter cells form within a mother cell. They can infect every mammalian cell except for non-nucleated erythrocytes \(^{8}\).

**Entry into host cells and formation of the parasitophorous vacuole**

The success of *T. gondii* as an intracellular pathogen is attributed to specialised adaptations that enable the tachyzoite to invade, reside and replicate within virtually all types of both phagocytic and non-phagocytic vertebrate cells \(^{17}\). Extracellular tachyzoites are highly susceptible to oxygen intermediates \(^{45}\), changes in pH \(^{46}\), and osmotic fluctuations, and are killed by specific antibodies in the presence of complement \(^{47,48}\).

Upon entry into macrophages, live tachyzoites secrete membranous vesicles (consisting primarily of surface proteins, particularly p30) that are reassembled to form a protein rich reticular network in the vacuolar space \(^{49}\). The modifications of the parasitophorous vacuole by this network allows the vacuole to evade microbicidal events, by enabling it to act as a protective interphase that prevents fusion of the vacuole with lysosomes \(^{50}\), or acidification of its contents \(^{46}\).
**Parasite invasion of the host gastrointestinal tract**

Following ingestion, the outer walls of the cysts or oocysts are disrupted by enzymatic degradation and the infective phase (bradyzoites and sporozoites respectively) are liberated into the intestinal lumen. They rapidly invade and multiply within the surrounding cells where they become tachyzoites. Thereafter, spread of *T. gondii* tachyzoites occurs by disruption of infected cells, followed by invasion of contiguous cells and via the blood stream and lymphatics primarily within cells. Widespread dissemination of the parasite occurs, and *T. gondii* can invade virtually all cells and tissues in the body, where they multiply intracellularly and cause cell death [51]. Cysts are formed particularly in the brain, heart skeletal muscle [11] and the retina.

**Host resistance to infection**

**Virulence of the Organism**

The virulence of the organisms plays an important role in the degree of cell destruction and the time of cyst formation. The less virulent strains produce less destruction in most cell lines, and tend to produce cysts earlier [38].

**Macrophage Activity**

The ability of the host to stop multiplication of *T. gondii* within newly invaded cells is dependent largely upon the activities of macrophages. The activities of macrophages in naïve hosts (i.e., hosts that have not experienced the invasion of toxoplasma previously), are inadequate to control the replication of the organism which can actively divide within the cytoplasm of the macrophage itself, destroying it before it can kill the parasite. In animals that have been exposed to *T. gondii*, macrophages have greater toxoplasmacidal activity.
Cell-mediated Immunity

Cell mediated immunity appears to be the principal resistance to *T. gondii* \(^{[52, 53]}\). In animals that have become immune to the parasite, T-lymphocytes have been found to secrete lymphokines that aid the macrophage in the destruction of the parasite. Both CD4+ and CD8+ T-cells play a role in resistance against acute infection. CD8+ cells are the primary mediators of this resistance both through cytotoxic activity \(^{[54]}\) and / or by the secretion of lymphokines such as Interferon γ (IFN γ) \(^{[55]}\).

It has been found that defects in cellular immunity are insufficient stimulus in their own right for reactivation of encysted organisms in healed retinal scars. Despite the severe defects in cellular immunity that occur in AIDS, patients have been reported with old healed *T. gondii* retinochoroidal scar \(^{[56]}\). However if reactivation occurs by other means cellular immunodeficiency may allow development of extensive disease \(^{[57]}\).

Role of cytokines

IFN γ, derived from T-lymphocytes \(^{[58]}\), appears to be a major mediator of host resistance against *T. gondii* \(^{[59]}\), possibly via a synergistic toxoplasma-cidal effect with Tumour Necrosis Factor (TNF) \(^{[60]}\). Amongst its many effects upon the immune system is the activation of macrophages \(^{[61]}\).

Humoral immunity

Infection with *T. gondii* triggers production of IgG, IgM, IgA, and IgE antibodies against both membrane and excreted/secreted antigens (ESA). Specific antibody in the presence of complement lyses extracellular tachyzoites \(^{[48]}\) and forms the basis of the Sabin-Feldman Dye test \(^{[47]}\)
Clinical Manifestations

Toxoplasmosis is considered in four categories:

1. Acquired in the immunocompetent patient,
2. Congenital,
3. Ocular,
4. Acquired or reactivated in the immunodeficient patient.

Acute acquired Toxoplasma infection in the Immunocompetent Patient

Only 10 to 20 percent of Toxoplasma infections in the adult are symptomatic [2]. In this group of patients toxoplasmosis most commonly presents as cervical lymphadenopathy, but any or all lymph nodes may become enlarged. Fever, malaise, night sweats, myalgia, sore throat, maculopapular rash, sparing the palms and soles, hepatosplenomegaly or atypical lymphocytes may be present [10]. The clinical picture may resemble infectious mononucleosis or cytomegalovirus infection (CMV).

In the immunocompetent patient the course of acute acquired toxoplasmosis is benign and self-limiting. Symptoms, if present, usually resolve within a few months, and rarely persist beyond a year. Rarely does an apparently healthy person develop clinically overt disseminated disease, with myocarditis, pneumonitis, or encephalitis, which can be fatal. Toxoplasmosis is difficult to diagnose clinically from the history, physical examination, and routine laboratory tests since its manifestations mimic many other diseases. The major diagnostic confusion occurs with Hodgkin's disease and the lymphomas [8].
**Congenital Toxoplasmosis**

Congenital toxoplasmosis is the result of an acute, but often subclinical maternal infection, acquired during pregnancy [29, 47]. Following the birth of a child with congenital toxoplasmosis, circulating maternal antibodies protect the foetus from infection in subsequent pregnancies [62]. There is a spectrum of clinical disease in patients with congenital *Toxoplasma* infection, retinochoroiditis, hydrocephalus, microcephaly, cerebral calcification, seizures, psychomotor retardation, hepatosplenomegaly, jaundice, rashes and fever [63]. Most infected newborns are asymptomatic at birth. Such infants may suffer no sequelae of the infection or may go onto develop retinochoroiditis, strabismus, epilepsy, psychomotor or mental retardation months to years later [64, 65]. Retinochoroiditis, has been found to be the most common clinical manifestation of congenital toxoplasmosis, present in 76% of cases [30]. Lesions are bilateral in 85% cases with a predilection for the posterior pole [66]. Neurological disorders were found in 51%, intracranial calcification in 32% and hydrocephalus or microcephaly in 26%. Toxoplasmosis as a cause of central nervous system disorders was found to be rare in the absence of ocular lesions [1].

**Ocular toxoplasmosis**

A retrospective study of 63 patients with active toxoplasma retinochoroiditis, found the average age of onset was 25 for the initial attack, (range 7 to 57 years). The average duration of each attack was 4.2 months (range 1 week to 2 years). The average number of active episodes was 2.7 per patient, (range 1 to 13) but 38% of the patients suffered only one attack and only 14% had more than four [67].
Toxoplasma gondii is the commonest cause of posterior uveitis in the immunocompetent patient, accounting for some 30-50% cases worldwide [12]. The Toxoplasma organism has a propensity for neural tissue, the lesion therefore starts in the retina, and it is only with ongoing inflammation that other tissues become involved in the clinical picture. The characteristic clinical lesion in both congenital and acquired toxoplasmosis is a focal necrotising retinitis [68] usually involving the inner retinal layers [63]. It appears as a whitish, fluffy lesion with surrounding retinal oedema and is classically juxtaposed to an old pigmented scar. Often the vitritis is so dense as to preclude accurate fundoscopy. There is often an associated posterior vitreous detachment with precipitates of inflammatory cells seen on the posterior hyaloid face [69]. Cuffs of perivascular exudates are seen along both retinal arteries and veins. O'Connor ascribes this phenomenon to antigen/antibody complex deposition [69] and has been able to duplicate this pathology experimentally in acutely infected rabbits subject to the intravitreal inoculation of soluble, cell free toxoplasmic antigens [70]. The underlying choroid is involved in a granulomatous reaction [71], and the sclera may also be involved in the underlying inflammatory response [63]. Lesions can vary greatly in size, are usually oval, and more frequently situated posterior to the equator. Central, bilateral lesions, especially those located at the macula, tend to be associated with the congenital lesions, although approximately 30% of congenital lesions have been found to be unilateral [72]. The acquired form tends to be unilateral, discrete, and solitary [73].

Symptoms of Ocular Toxoplasmosis

Symptoms are present in over 90% of patients with active Toxoplasma retinitis [63]. The majority of patients present with a history of floaters; however patients can also
present with reduced visual acuity due to foveal involvement with retinochoroiditis or from media opacities due to the overlying vitritis. Metamorphopsia due to serous detachment of the macula secondary to deep retinal lesion can occur. Reduction of visual acuity can also be secondary to cystoid macular oedema or cataract formation in chronic disease.

**Acquired Ocular Toxoplasmosis**

Whilst retinochoroiditis can occur with acquired toxoplasmosis\(^{[26, 74, 75]}\), it has been assumed that this is rare in the immunocompetent person. In his treatise *Uveitis and Toxoplasmosis* Perkins states, “It is probable that the vast majority of cases of toxoplasmic uveitis in adults and children are recurrences of congenital infection”\(^{[76]}\). Perkins’ literature review found uveitis described in 3.1% cases of acquired toxoplasmosis reported, most commonly in association with toxoplasmic encephalitis\(^{[62]}\). Others reported that less than 1% of patients with acute acquired toxoplasmosis develop retinitis\(^{[77]}\).

On the basis of the *Toxoplasma* serology of patients with recently acquired toxoplasmosis it seems likely retinochoroiditis occurs more commonly than previously thought\(^{[78-80]}\) and should be considered in the differential diagnosis of unilateral focal retinochoroiditis in all age groups\(^{[81]}\). Acquired ocular toxoplasmosis has been estimated to be bilateral in approximately 16% cases\(^{[82]}\). In a retrospective analysis of the immune status of mothers of patients with ocular toxoplasmosis, others too have concluded that the acquired pattern of ocular toxoplasmosis might be more frequent than had hitherto been estimated\(^{[83]}\). Furthermore active retinochoroiditis may be the only clinical abnormality in acquired toxoplasmosis\(^{[84]}\) and may also occur years after the acute infection\(^{[73, 74, 84-86]}\). In an outbreak of systemic toxoplasmosis in Georgia in 1977, there was no
evidence of ocular toxoplasmosis after one year, however, at four years follow up one patient showed evidence of ocular disease [73]. In the largest series of acquired toxoplasmic retinochoroiditis occurring within a single outbreak, twenty-one eyes of 20 patients presented with retinal lesions, many occurring within macular-peripapillary region [87]. Increasing prevalence of ocular toxoplasmosis with age has been found in a Brazilian study; 20% of adults had toxoplasma retinochoroidal lesions. Most of these lesions were attributed to acquired infection as only 1% of cord blood samples in that population were positive for IgM [88].

**Toxoplasmosis in the immunocompromised host**

Toxoplasmosis has emerged as an important opportunistic pathogen in immunocompromised patients capable of causing rapidly fatal disseminated disease. Cell-mediated immunity has a crucial role in the control of *Toxoplasma* infection [89]. When this defence system is compromised by diseases of the lymphoreticular system, intensive tumour radiotherapy, or by immunosuppressive therapy, the organism is free to proliferate in an uninhibited fashion [90, 91]. Although immunodeficient patients are more susceptible to serious disease if they acquire *Toxoplasma* infection, it is likely that clinically apparent toxoplasmosis in these patients is most often a consequence of reactivation of latent infection [91-93]. The threat to this population appears to be greatest in those patients with Hodgkin’s disease, organ transplants or AIDS. Neurological syndromes consistent with diffuse encephalopathy, meningoencephalitis, or cerebral mass lesions predominate [89, 91, 94]. Overt myocarditis or pneumonitis may also be present [94] [8]. Many concomitant infections with DNA viruses are also seen [89, 91, 94] [95] [96]. The threat to this population appears to be greatest in those patients who have received immunosuppressive therapy for underlying haematological disease, most notably in
those patients with Hodgkin’s disease [91, 94, 97], and in patients with leukaemia [91]
[94] [98]. It is also seen in patients treated for other tumours [99] as well as in those
patients who are immunosuppressed following organ transplantation [99, 100] [101] [4],
or collagen-vascular disorders [91, 102].

Ocular Toxoplasmosis in the immunocompromised

In immunocompromised patients Toxoplasma infections in the eye have been
reported only rarely, primarily in patients with disorders characterised by cellular
immunodeficiency [103]. When ocular toxoplasmosis does occur in
immunocompromised patients or if immunosuppressive therapy is given to a patient
with active disease, widespread tissue destruction can occur. An association
therefore could be expected between ocular toxoplasmosis and immunosuppression,
particularly in the case of injudicious use of steroids which exert profound effects on
lymphocytes and disruption of phagocytosis whilst exerting less effect on antibody
producing plasma cells [104].
The immunosuppressive effect of large doses of steroids (when administered
without concomitant antimicrobial therapy), particularly sub-tenons injections of
depot steroids, has been associated with deterioration of ocular toxoplasmosis [24, 90,
105, 106]
The association between steroids and the recurrence of inactive toxoplasma lesions
is less clear. It has been reported that combined use of steroids with irradiation
allowed reactivation of retinochoroiditis in animal models [107]. Conversely others
have found that rabbits with chronic toxoplasmosis did not develop recurrence or
worsening of ocular disease during or after treatment with corticosteroids [108, 109].
In humans the reactivation of retinochoroiditis in a patient treated with steroids and
azathioprine after renal transplantation has been reported [110]. Others have been
unable to confirm the causal relationship between corticosteroid use and initiation of disease recurrence [111]

Ocular toxoplasmosis in the immunocompromised can present difficulties, both in the diagnosis and treatment of the condition. The appearance of the lesion is frequently atypical, active lesions may be bilateral and multifocal, the majority do not arise from the borders of pre-existing scars, suggesting that the lesions result from acquired infection or by dissemination of organisms from non-ocular sites [112]. The condition can be mistaken for acute retinal necrosis [24] and cytomegalovirus retinitis [106]. Delay in accurate diagnosis leads to extensive destruction of the retina. Widespread bilateral retinal necrosis seen in a patient with a lymphoproliferative disorder whilst receiving prolonged systemic steroid therapy has been reported [113]. Histopathological examination revealed tachyzoites and cysts with little inflammatory reaction. Widespread, chorioretinal atrophy and acute bilateral retinochoroiditis associated with thymoma have both been reported [114, 115]. Cases of acquired ocular toxoplasmosis without concomitant CNS involvement has been reported in patients with Hodgkin’s disease who were being treated with chemotherapeutic agents. [116]. Macular lesions in these patients may be so severe as to spread to involve the entire posterior pole. Diagnosis may be made only at post-mortem, as diagnostic vitrectomy maybe inconclusive, and Toxoplasma serology negative [117]. In none of these cases were there retinochoroidal scars at the time of the disease developing.

**Toxoplasmosis and AIDS**

Fever, rash, myocarditis, and pneumonitis are all systemic manifestations of *T. gondii* in the immunocompromised [118]. Unlike other immunocompromised patients, patients with AIDS have a particular predilection for the development of
Toxoplasma encephalitis [91, 94, 119, 120], the most common nonviral intracranial infection among patients with AIDS [6, 93, 120]. This usually results from reactivation of organisms causing latent infection [6, 91]. Nearly 80% of AIDS patients show CNS pathology at autopsy [121], approximately 32% of patients with latent *Toxoplasma* infection will develop toxoplasmic encephalitis during the course of their HIV infection [122]. CD4+ count <100/ml, positive *T.gondii* serology [120], and lack of prophylaxis predispose to the disease [122]. Difficulties exist with current diagnostic tests for toxoplasmosis in this patient group. The majority of AIDS patients fail to mount an antibody response indicative of acute infection with *Toxoplasma* [6, 119, 123]. The absence of specific IgM antibody or rise in the IgG antibody titre to *T.gondii* does not exclude this condition in the immunocompromised host [120, 123, 124]. Despite elevated total immunoglobulin levels, serological responses to specific infections could be impaired as a result of the helper T-cell deficiency [124].

**Ocular Toxoplasmosis in HIV and AIDS**

As with other immunocompromised patients, ocular toxoplasmosis has been reported infrequently [125, 126] with 1-3% of ocular infections in AIDS patients estimated to be due to *T.gondii* [127]. Nonetheless *Toxoplasma* retinochoroiditis is the second most common infectious cause of ocular lesions in the HIV population [128]. About 10%-20% of patients with intracranial toxoplasmosis have eye involvement [127]. Although not a criterion for the diagnosis of AIDS ocular toxoplasmosis frequently occurs early in the course of HIV disease and may be the initial ocular infection in patients, preceding serological diagnosis of the HIV disease [128-130]. Retinochoroiditis usually does not occur at the borders of pre-existing scars [112, 126, 127, 130-132], and frequently starts adjacent to retinal blood
vessels, suggesting that the parasites reach the eye via the blood stream possibly from non-ocular sites [112].

Retinochoroiditis may represent cases of newly acquired *T. gondii* infection [133, 134]. Elevated anti-toxoplasma IgM has been found in 12% of AIDS patients with ocular toxoplasmosis [128]. Retinochoroidal scars consistent with healed *Toxoplasma* lesions have been seen in AIDS patients at autopsy without evidence of reactivation [56]. *Toxoplasma* cysts have been seen in the brain of an AIDS patient at autopsy without evidence of reactivation [125]. Therefore whilst the severe immunodeficiency associated with AIDS allows proliferation of the *Toxoplasma* organism, it alone may be insufficient stimulus for the reactivation of the encysted organisms [127].

As *T. gondii* retinitis may be bilateral it is essential to establish the diagnosis as rapidly as possible as the disease is often fulminant and aggressive [126]. Immunodeficiency and delays in diagnosis and treatment potentiate widespread *T. gondii* tissue destruction and visual loss may be rapid. Both the aqueous coefficient and serological tests are unhelpful in making the diagnosis of *T. gondii*. [128, 134, 135] The latter may remain negative throughout the clinical course of the disease [136].

There are several clinical manifestations. Lesions may be unilateral [137], but are frequently bilateral. Lesions described include single discrete lesions, multifocal discrete lesions [31, 112], diffuse areas of retinal necrosis, similar in appearance to acute retinal necrosis [112, 131], a pattern of miliary retinitis [136], panophthalmitis [135], and even optic neuritis. [138]. There may be associated retinal detachment [7, 112]. Prominent inflammatory reactions in both the vitreous and anterior chamber are common [112, 134] and in proportion to the patient's CD4+ count. [135] The disease
can not only be confused with CMV retinitis [106, 138] but can also co-exist with it. [112, 128, 132, 139-141] T. gondii retinal lesions in patients with AIDS are usually extensive and can cause visual impairment if left untreated [130, 131, 134, 138]. Early and appropriate treatment of ocular toxoplasmosis in AIDS patients is vital. Not only is the ocular prognosis good [128], but ocular disease often co-exists with life threatening cerebral involvement [127, 128, 140], and may well precede neurological signs [112, 127, 130, 133, 141], none of which are pathognomonic of T. gondii involvement in the CNS [121]. The correct identification of T. gondii as the cause of intraocular inflammation should therefore alert the ophthalmologist of the need for CNS investigation. In general, early diagnosis and aggressive and prolonged therapy with pyrimethamine and sulphadiazine results in dramatic clinical and radiological response and reduction in mortality associated with this infection in AIDS [121, 124].

**Histopathology of Ocular Toxoplasmosis**

T. gondii retinochoroiditis was first recognized in 1952 [142]. Organisms were isolated from an enucleated eye in 1954 [143].

The characteristic lesion of ocular toxoplasmosis is a focal necrotising retinochoroiditis [69, 144] consisting of a zonal granuloma, the retina, pigment epithelium and choroid exhibit varying degrees of coagulative necrosis. The margins of the retinochoroidal lesion are usually well defined, wherein the retina is necrotic and the architecture is completely destroyed. There is an abrupt change into chronically inflamed but non-necrotic retina in which the tissue architecture is intact [63, 144, 145]. Toxoplasma cysts have been observed in all retinal layers [113, 117], including the pigment epithelium [113]. They are usually located in the inner retina, particularly the ganglion cell layer [146, 147], and may be present either adjacent to
scars or within the area of retinal necrosis, together with numerous dispersed pigment epithelium melanin granules [145, 148]. Cysts may also occur in the region of the optic nerve head [147]. No evidence that *Toxoplasma* cysts provide the foci for inflammatory cell attack has been found [71]. Phagocytosis of photoreceptor outer segments by macrophages is observed [71]. The choroidal inflammatory reaction is secondary to the parasitisation of the retina and retinitis [82, 144, 149], and may be either a diffuse granulomatous or a non-granulomatous mononuclear cell infiltrate [43, 113, 117, 148]. Severely affected eyes exhibit vasculitis and inflammatory cell invasion into the vitreous. Parasites have been observed in the vitreous in a vitrectomy specimen [150].

**Histopathology of Toxoplasma retinochoroiditis in the immunocompromised**

In AIDS a more diffuse coagulative necrosis occurs because of the decreased ability of the immunocompromised host to contain the proliferation of organisms and hence the lesions in AIDS patients tend to be larger than in the immunocompetent host [7, 151]. Increasing numbers of encysted and free tachyzoites admixed with few necrotic cysts are seen in the necrotic retina [112, 132]. There is an absence of a well-developed granulomatous choroiditis and significant inflammatory cell infiltration of the retina [126]. Cysts have also been found in RPE [7], the sclera [135], and the iris [152]. Tachyzoites have been found in the optic nerve [112, 138], the choroid [112], clustered around blood vessels [112] and in the anterior vitreous [112].

**Chronicity and Recurrences**

After an active infection the disease enters a chronic stage when tissue cysts are formed mainly in the brain, skeletal and cardiac muscle, and the eyes [153]. In these
tissues cysts will form as early as 8 days after infection [154]. These cysts tend not to evoke an inflammatory response [71].

The signal for cyst formation is not clear, but it may be a response to antibodies against T. gondii [155].

Pathogenesis of tissue damage in T. gondii retinochoroiditis

The pathogenic mechanisms whereby T. gondii tissue cysts reactivate and the processes by which retinochoroiditis causes damage to retinal and adjacent tissues is the subject of controversy. They include invasion and parasitisation of retinal tissues by the Toxoplasma organism and localized tissue damage by a complex associated immune response [156]. The pathogenic mechanisms that have been suggested include;

A) Local invasion of tissues by T. gondii organisms
B) a toxic effect of the organism or its products
C) damage as a result of an “innocent bystander” mechanism in which active phagocytosis and inflammatory cell lysis result in damage to surrounding tissues
D) a hypersensitivity reaction to toxoplasma organisms and
E) damage as a result of auto-immune mechanisms of tissue destruction

Local invasion of tissues by T. gondii organisms

Direct invasion of retinal cells by tachyzoites is probably the most important cause of tissue damage [115, 52, 57]. Upon entry into the host cell the organism replicates within a “parasitophorous vacuole” which probably incorporates host–cell structures [16, 146]. Perhaps as a result of this, cyst walls have a lack of antigenicity and engender little if any inflammatory reaction [71, 147, 157]. It is likely that such cysts remain dormant in the retina for long periods [3].
It is proposed that *Toxoplasma* cysts eventually rupture as replication of organisms occurs. This allows dissemination of free parasites which invade adjacent cells, which causes the recurrent inflammatory episodes of toxoplasmic retinochoroiditis [158]. Evidence that such an event occurs is circumstantial; evidence of *Toxoplasma* cyst rupture has been obtained from cell culture experiments [38] and retinal parasitisation by a related organism, *Besnoita jellisoni*, indicates that such cysts may undergo spontaneous rupture and thereby induce an inflammatory response [159]. In addition the extensive destruction of the retina has been demonstrated in the absence of any significant infiltration by inflammatory cells [113]. In tissue culture infected with *T.gondii* organisms there is invasion of cells by the organism, rapid proliferation within the cytoplasm, eventual rupture of the cell membrane with release of the *T.gondii* organisms and subsequent invasion of other adjacent cells [52]. The factors determining the occurrence and timing of disease recurrence is unknown. Both the virulence of the parasite and the integrity of the host immune responses probably play important roles.

*A toxic effect of the organism or its products*

It has been argued that a cytolytic and necrotising action of the parasite can cause necrotising retinitis in the absence of significant inflammatory response [117]. A toxin derived from the peritoneal exudate of *Toxoplasma* infected mice has been reported to cause severe retinal damage when inoculated into the vitreous of the rabbit eye [160]. However toxic components derived from the inflammatory cells of the mice may have been responsible for the retinal damage.

Staining outside the confines of the encysted parasites has been seen when *Toxoplasma* antigens were labelled by immunofluorescence. A diffusible antigenic
toxin was postulated \cite{161}. Others failed to reproduce these findings using analogous labelling techniques \cite{162}. Moreover, ultrastructural studies performed in the same model failed to reveal any morphological evidence of retinal destruction in the immediate vicinity of the *Toxoplasma* cyst \cite{146}.

*Active phagocytosis and inflammatory cell lysis result in damage to surrounding tissues*

Inflammatory cells contain lysosomal enzymes whose release may result in damage to surrounding cells as a result of their being juxtaposed to the inflammatory process \cite{163}. Such a phenomenon has been observed in electron-microscopic studies in which inflammatory cell lysis has resulted in neighbouring retinal cell damage \cite{71}.

*Hypersensitivity reaction to toxoplasma organisms*

The histopathology of severe toxoplastic retinochoroiditis is classically that of a granuloma \cite{144,164}. Subcutaneous inoculation of toxoplasmin antigen derived from *Toxoplasma* gives rise to a delayed hypersensitivity reaction in the majority of infected patients \cite{165}. Intravitreal injection of a foreign protein into a previously sensitised animal gives rise to such a response within the uvea \cite{166}. For these reasons it has always been believed that cyst rupture results in intra-ocular inflammation as a result of delayed hypersensitivity to *Toxoplasma*.

A number of experimental studies have been carried out in which *Toxoplasma* antigen has failed to elicit recurrent inflammation within eyes previously, experimentally infected with *Toxoplasma* \cite{167-169}. It has been concluded from these experiments that hypersensitivity to *Toxoplasma* is unlikely to be responsible for the recurrent eye disease process. However, delayed hypersensitivity to *Toxoplasma*...
antigen could be responsible for perpetuation of the inflammatory response once
cyst rupture has taken place.

Lymphocyte transformation assays against *Toxoplasma* in patients with ocular
toxoplasmosis provide evidence of a specific cell mediated immune response against
the organism [170].

*Damage as a result of autoimmune mechanisms of tissue destruction*

Because of the selective photoreceptor destruction, it has been speculated that
autoimmune reactions may play an important role in the tissue destruction seen in
ocular toxoplasmosis [31]. It is probable that in recurrent retinochoroiditis there are
not only *T. gondii* antigens, but antigens of retinal origin as well [52, 171]. “S-antigen”,
a soluble antigen derived from photoreceptor outer segments has been characterised
[172], and appears to have an important role in certain types of chronic and recurrent
ocular inflammatory conditions. This antigen is capable of inducing cell-mediated
immune responses, and in sensitised individuals its release into the tissues may be
accompanied by massive infiltration of both lymphocytes and macrophages.
Exaggerated cell-mediated responses to S-antigen may be present in patients with
recurrent *T. gondii* retinochoroiditis [170, 173].

Studies on peripheral blood from patients with toxoplasmic retinochoroiditis have
indicated that both humoral [174-176] and cell mediated [170, 173, 177] immunity against
retinal antigens exist. In animal studies of toxoplasmic retinochoroiditis where the
inflammatory response has been discrete and not overwhelming, selective
destruction of outer retina with sparing of inner retina has been documented [157, 178-
184]. In studies on mice congenitally infected with *T. gondii* such a phenomenon was
commonly observed with considerable variation in the degree of inflammatory cell
infiltration [71, 147, 157, 162]. These histological appearances were analogous to those
of experimental allergic uveoretinitis in which the outer retina is selectively destroyed following the inoculation of S-antigen [172].

Other studies to investigate the pathogenesis of the outer retinal destruction failed to detect any anti-retinal antibodies in the sera from mice with retinochoroiditis [185]. On the other hand ultrastructural studies have indicated that in eyes with histological evidence of mild disease the outer retinal damage was caused predominantly by macrophages; suggesting cell mediated hypersensitivity (Type IV) [71, 162]. These cells were commonly encountered phagocytosing the photoreceptor elements of the outer retina. Lymphocyte transformation assays against retinal antigens using splenic lymphocytes from animals of the same model provided limited evidence suggestive of cell mediated immune responses against retinal antigens in infected mice as compared with controls [186].

When cellular immune responses to retinal S antigen and a toxoplasma extract in patients with toxoplasma retinochoroiditis and other uveitic entities have been compared. Only a small number of toxoplasma patients showed positive cellular immunity against retinal S antigen, which was not significantly different from the non-toxoplasma group. Secondly, the cellular response to toxoplasma antigens was not different in the two groups, which indicates that neither humoral nor cellular reactivity against toxoplasma antigens in peripheral blood samples has any clinical diagnostic value. Arguments favouring a role for a local inflammatory response against toxoplasma organisms comes from the observation that a majority of patients with toxoplasma retinochoroiditis exhibit intraocular antibody production against the organism [187].
Summary of Pathogenesis

The retinochoroiditis caused by *Toxoplasma* within the eye appears to result from a complex combination of varied mechanisms. Invasion of retinal cells by tachyzoites is probably the most important cause of tissue destruction. Release of free parasites from intra-retinal cysts also promotes an inflammatory response. Current evidence indicates that a humoral immune response is mounted against the parasite, and that this may be combined with a specific cell mediated response. A probable immune response directed towards the retina compounded by non-specific damage as a result of inflammatory cell lysis complicates the picture still further.

Laboratory Methods used for the Diagnosis of Toxoplasmosis

Serology

Serological evidence of infection is important in making the diagnosis of *Toxoplasma* infection. Serological investigation must utilize a highly sensitive IgG assay, as the absence of circulating, specific IgG makes the diagnosis unlikely [188]. The presence of specific IgG and compatible clinical findings form the basis for the diagnosis in most cases [63]. However because the prevalence of antibodies to *Toxoplasma* is high in certain communities, and because high antibody titres can persist for many years in otherwise healthy individuals, the presence of anti-*Toxoplasma* antibodies in the peripheral blood has a low specific diagnostic value in ocular disease [189]. In humans *Toxoplasma* seropositivity increases with age. In France, the prevalence of anti-*Toxoplasma* antibodies is greater than 90% by the fourth decade.

In ocular toxoplasmosis, low titres of IgG antibody to *T.gondii* are usual [63]. There is no relationship between the degree of positivity of the dye test and the diagnosis
of ocular toxoplasmosis \[82\], during active disease no typical change of the titres occur \[189\].

**Serological Diagnosis in different Patient Groups**

**Acute acquired primary Toxoplasmosis**

Detection of IgM antibodies, is the method of choice in patients suspected of suffering from acute, primary infection. In most patients high levels of IgM and IgG antibodies will be found at presentation \[190\]. Seroconversion or antibody titre rises can be demonstrated less frequently. For this purpose the indirect haemagglutination test \[191\] and ELISA \[192\] are most suitable since maximum antibody titres are reached only 5-7 months after infection in these tests.

**Infection in the immunocompromised host**

Serological diagnosis of *T. gondii* infection can be difficult in AIDS and other immunosuppressed patients. Unlike immunosuppressed cancer patients who consistently have high and diagnostically rising antibody titres when infected with *T. gondii* \[91\], AIDS patients may not have diagnostically significant serologic findings \[134\]. Despite elevated total immunoglobulin levels in AIDS patients, serological responses to specific infections can be impaired as a result of the CD4+ T-cell deficiency. The absence of specific IgM or rise in the IgG titre to *T. gondii* does not exclude this pathogen as the causative agent of disease \[91, 124\].

Although clinical disease is infrequent in the majority of immunocompromised patients with reactivation of latent infection, encephalitis is a well-known complication in the severely immunocompromised, particularly AIDS patients \[120, 193\]. Reactivation of a latent infection has to be diagnosed by a significant antibody titre rise or isolation of *T. gondii* from patient specimens \[194\].
In AIDS patients, the ratio of titres of the agglutination test to titres of the dye test seem to be more predictive of active *Toxoplasma* encephalitis than either test alone [6].

**Classification of Antibody Tests**

Antibody tests for toxoplasmosis can be classified into two major groups:

A) those using whole intact organisms

B) those using disrupted parasites as an antigen source

Antigens used in toxoplasma diagnostic assays come from either the plasma membrane or the organism's cytoplasm. In early infection the host’s immune response is directed towards the membrane antigens whereas the cytoplasmic antigens become progressively more important as antibody targets in chronic infection. Assays utilizing whole organisms as an antigen source are most reactive in early infection while tests incorporating disrupted parasites produce titres which rise more slowly during the course of the infection but persist for a greater time [195].

**Antibody tests using whole intact organisms**

A) *The Sabin-Feldman Dye Test*

Live *Toxoplasma* organisms are incubated with the serum to be tested and complement for one hour after which time a vital stain, alkaline methylene blue is added [196]. If anti-*Toxoplasma* antibody is present in the serum, it will cause complement-mediated cytolysis of the parasites and the organisms are thus unable to retain the dye [195].

Serial dilutions are made of the serum to be tested, and the titre is the dilution of serum at which greater than 50% of the organisms remain unstained. A positive control produces a dye test titre of 1:256 [195].
Antibodies appear 1 to 2 weeks after the initial infection, reach a peak titre in 6 to 8 weeks, and decline over 1 to 2 years. Low titres probably persist for life. Titres greater than 1:4 are considered positive, and may rise to greater than 1:1024 in an acute systemic infection [63].

The Dye test is sensitive and specific, and is the reference test against which all other procedures are evaluated. Titres of the dye test do not correlate with the severity of the disease nor with the activity of the ocular disease [189]. Low titre false positives are seen in *Hammondia hammondii* infection or following blood transfusion [195]. False negative results are extremely rare but have been reported in immunosuppressed patients [119]. Failure of dye test titres to rise to greater than 1:1024 has been reported in immunodeficient patients with active CNS toxoplasmosis [119, 120, 133, 197].

**B) Fluorescent antibody Test (FAT)/ Indirect Fluorescent Antibody (IFA) Test**

Outside the U.K. the IFA is the most widely used procedure for the diagnosis of toxoplasmosis [195]. The test uses killed rather than live organisms. Killed tachyzoites are fixed on to a slide and incubated with dilutions of the test sample. Using appropriate fluorescein tagged anti-human serum, IgG and IgM antibodies can be detected [196].

*IFA test for IgM*

The IFA test for IgM antibodies is useful in the diagnosis of acute acquired infection in both the normal and immunocompromised patient [89] IgM antibody titres appear rise rapidly as early as 5 days after infection and then fall and disappear within a few weeks to months. Occasionally they may persist for years, but usually at low titres of < 1:20. [89]. During the acute infection, IgM titres may vary greatly, but only a
fourfold or greater rise or an extremely high titre (1:160 or greater) establishes the presence of acute infection. A low titre does not exclude acute infection [198].

**IFA test for IgG**

IgG titres rise more slowly than those of IgM, taking 2 months or more to reach a peak. After 4-6 months the IgG titre begins to fall but because high antibody titres of IgG have been known to persist for years an isolated high titre does not establish an acute infection [11]. Some antibody usually persists indefinitely. As with the IgM IFA a fourfold rise in IgG antibody titres between serum samples run in parallel establishes the diagnosis of acute infection [198].

**False Positive Results**

IgM fluorescein conjugates can give false positive reactions due to non-specific “capping” of the trophozoites [195]. Antinuclear antibodies may cause false-positive IgM and IgG anti-*Toxoplasma* titres [199]. Rheumatoid factor may also cause false positive IgM reactions [200, 201]. This occurs because rheumatoid factor (which is an IgM anti-IgG) can bind to specific anti-*Toxoplasma* IgG. These false reactions can be avoided by using gel filtration to separate IgM from IgG antibodies before testing, or preabsorption of serum with a preparation containing staphylococci and streptococci which eliminates nonspecific IgM reactions by removing all IgG [200, 202].

**False Negative Reactions**

Fluorescent antibody tests for IgM are less sensitive than ELISA or agglutination assays and therefore results should be confirmed using more sensitive and specific assays [195]. False negative results may occur in up to 40% samples [192] due to competitive inhibition by high levels of *T. gondii* specific IgG antibodies [203].
C) Direct Agglutination Test

Recently a direct agglutination test has been described to differentiate acute and chronic disease.

**Antibody Tests using Disrupted parasites as antigen source**

A) Complement Fixation Tests

The technique is of limited value. The complement fixing antibodies measured by this test appear weeks later in the disease and decline earlier than the antibodies measured by the dye test, although they may remain positive for many years. Therefore a negative test does not rule out the possibility of past infection with *T. gondii*, and a positive result does not establish the diagnosis of acute infection.

B) Enzyme-linked Immunosorbent Assay (ELISA)

The ELISA test for *Toxoplasma* antibodies is believed to be as sensitive as the dye test [154] and can be used to detect either IgG, IgM and IgA toxoplasma antibodies, as well as to detect free *Toxoplasma* antigen and circulating immune complexes containing *Toxoplasma* antigen [8, 204].

The Double-sandwich IgM-ELISA

A more sensitive and specific test than both the conventional IgM ELISA and the IgM IFA tests [205-207]. One study found that 93% of sera that were obtained from adults with recently acquired toxoplasmosis that were negative in the IgM-IFA test were strongly positive in the double-sandwich IgM-ELISA [8].

The double sandwich enzyme linked immunoassay test eliminates IgM specificity problems associated with the immunofluorescent antibody test because only IgM of the test serum adheres to plates pre-coated with anti-IgM antiserum [208, 209].
C) Latex Agglutination Test

The slide agglutination test has been compared to the dye test for the detection of *Toxoplasma*-specific IgG and has proven to have a sensitivity of 98.7% and a specificity of 95.8%. It is therefore suitable as a screening assay for toxoplasmosis, but not as a replacement for the dye test. It is recommended that sera from the immunocompetent patients showing positive agglutination at ≥1:16 and at neat sera for the immunocompromised patients are examined by the dye test [195].

D) Indirect Haemagglutination Assay

The test becomes positive 2-4 weeks after acute infection, and antibody titres remain elevated for many months. False negative results are not infrequent, and so whilst it may be used as a screening test, the haemagglutination test is less helpful in diagnosing toxoplasmosis [63, 195]

Local Antibody Production in Toxoplasmosis

Desmonts postulated that in patients with purely a recurrence of ocular toxoplasmosis, local antibody production against the organism would occur and therefore the local titre will be greater than that found in the circulation. Such a finding would be of use where diagnostic problems arose with atypical lesions. To demonstrate local (intraocular) production of antibody, the specific antibody in the eye is measured relative to the total amount of immunoglobulin in the eye. This led Desmonts to calculate the antibody coefficient (C). The formula for determining the value is:

\[
C = \frac{\text{Antibody Titre}_{\text{aqueous humour}} \times \text{Immunoglobulin}_{\text{serum}}}{\text{Immunoglobulin}_{\text{aqueous humour}}} \]

57
The normal range is from 0.5 to 2.0 [63, 210]. A coefficient from 2 to 7 is compatible with local production of specific antibody, but a coefficient above 8 is highly suggestive of local antibody production [31, 63]. Eyes with inactive scars have a normal coefficient, indicating that recent antigenic stimulation is necessary for the production of local antibody. The coefficient may take three weeks to become positive in eyes with active disease [210].

Use of ELISA techniques increases the sensitivity of this method [204, 211, 212]. Modified enzyme immunoassay demonstrates IgG class antibodies are produced locally within the eye in cases of Toxoplasma retinochoroiditis. This increase in intraocular levels of anti-Toxoplasma antibodies has been shown not to be as a result of either increased diffusion or as a result of polyclonal antibody stimulation [213]. Immunoblot analysis demonstrates intraocular production of both IgG and IgA anti-Toxoplasma specific antibodies in patients with Toxoplasma retinochoroiditis [214]. It would appear that IgG is the major immunoglobulin class involved in the intraocular humoral response against the parasite during recurrent ocular toxoplasmosis. When detection of IgA is combined with determination of IgG and detection of T. gondii DNA it is possible to identify 91% of patients with a final diagnosis of ocular toxoplasmosis [215]. Aqueous derived antibodies demonstrate different recognition patterns to those derived from serum strongly suggesting intra-ocular immunoglobulin synthesis. Others have demonstrated different avidities in aqueous compared to serum derived IgG from patients with Toxoplasma retinochoroiditis [216]. The differences between aqueous and serum antibody response appear to occur in patients with recurrent chronic Toxoplasma retinochoroiditis but not in patients with acute acquired retinochoroiditis [80].
A major disadvantage of aqueous humour analysis was found to be the occurrence of false negative antibody coefficients associated with massive blood aqueous barrier breakdown [187]. Under such circumstances, high serum titre of antibodies can mask intraocular antibody production. The coefficient has also been found to be close to normal in active generalised toxoplasmosis, both congenital and acquired, associated with active ocular lesions [210].

Furthermore elevated levels of intraocular anti-Toxoplasma antibodies can occur in patients with uveitis not due to Toxoplasma but who have positive Toxoplasma serology. The inflammation in the diseased eye increases the globulin concentrations, however the coefficient remained close to 1.0 [210]. Kijlstra too stresses that an isolated analysis of aqueous is worthless since the presence of antibodies or antigens in the aqueous may be due to breakdown of the blood aqueous barrier [188].

Development of positive coefficients appears to be infrequent in AIDS patients with Toxoplasma retinochoroiditis [128].

**Demonstration of T.gondii directly in patients’ specimens**

A definitive diagnosis of acute toxoplasmosis can be made by the demonstration of tachyzoites in tissues or body fluids [198]. The recognition of trophozoites in histological sections can be difficult [217], but can be facilitated by using Giemsa or Wright stains. Immunofluorescent staining has been used and may facilitate identification of organisms, offering higher sensitivity and specificity [218].

**Isolation of T. gondii**

Isolation of the organism from body fluids establishes the diagnosis of toxoplasma infection. Usually this method involves intraperitoneal inoculation of the mouse [3],
or in tissue culture cells [194, 219]. Similar to the demonstration of cysts directly in patient’s specimens, mere isolation of the parasite from tissues has to be interpreted with caution, since it may simply reflect the presence of cysts. This neither proves acute infection, nor excludes it [220]. Inoculation of mice is usually considered to be the most sensitive method of isolating *T. gondii*, however results are obtained only after several weeks and hence there is often a delay in the diagnosis [220]. Other studies [194, 219, 221] have shown human diploid fibroblast cells to be an alternative to mice for isolating *T. gondii*. The sensitivity is similar, and the results are obtained more rapidly.

**Isolation of T. gondii from the eye**

Isolation of *T. gondii* has been reported from sub-retinal fluid [222]. Culture of aqueous samples has proved to be unsuccessful [82, 188], and difficult from vitreous [82].

In AIDS patients however, parasites have been found in the vitreous [133, 134, 223] and aqueous humour [112].

**Use of the Polymerase Chain Reaction in the diagnosis of toxoplasmosis**

PCR amplification of sequences within the B1, P30 and ribosomal genes of *T. gondii* have been assessed in a variety of tissues, in both the immunocompetent and immunocompromised patient. Blood [224-227], bronchoalveolar lavage [228], cerebrospinal fluid [225, 226, 228-232], liver [228], urine [225], amniotic fluid [231, 233, 234], cardiac tissue [235], cerebral tissue, [228, 236, 237], have all been investigated using these methods.
Polymerase Chain Reaction

The polymerase chain reaction was first described in 1985 [238]. Repeated cycles of oligonucleotide-directed DNA synthesis carry out in-vitro replication of target nucleic acid sequences. In the presence of deoxynucleotide triphosphates in a suitable buffer system, DNA polymerase synthesizes DNA from a molar excess pair of oligonucleotide primers that are complimentary to opposite strands of DNA, flanking the region to be amplified.

The use of a thermostable DNA polymerase (Taq polymerase) has allowed automation of the heat cycling process. The detection of characteristic nucleic acid traces in a clinical specimen is presumed, in most cases to be evidence of concurrent (or recent past) infection. The reaction consists of a series of steps;

Denaturation

Normally DNA in solution is double stranded. The hydrogen bonds that bind the purine (adenine and guanine) and pyrimidine (thymine and cytosine) base pairs of the complementary DNA strands can be broken by heating. Such denaturation produces two complimentary single strands. The melting temperature is dependent upon the buffer conditions but also, more importantly, on the DNA sequence which therefore may be relevant in determining optimum PCR conditions.

Annealing

The denatured sample DNA is in solution with an excess of the two complimentary primers. Upon cooling DNA will re-anneal. In most cases it will be the oligonucleotides that will anneal to their complimentary regions as they are in great excess. If the annealing temperature is quite close to the melting temperature then the PCR is extremely sensitive as no base mismatches will be tolerated.
**Amplification**

The reaction mix is then heated to the optimum temperature for the activity for the Taq polymerase, around 72°C and, using deoxynucleotide triphosphates complementary DNA is synthesized. A DNA molecule has polarity. One end of the chain has a 5'-OH group and the other a 3'-OH group that is not linked to another nucleotide. DNA is synthesized in the 5' to 3' direction; the chain elongation reaction catalysed by DNA polymerase occurs by means of a nucleophilic attack of the 3'-OH terminus of the primer on the innermost phosphorus atom of the incoming deoxyribonucleoside triphosphate. A phosphodiester bridge is formed and pyrophosphate is concomitantly released. The subsequent hydrolysis of the pyrophosphate drives the reaction forward [239].

During the first cycle, DNA synthesis is restricted only by time. Similar processes occur in both strands. One of the strands of each daughter molecule is newly synthesized, whereas the other is derived from the parent molecule. As proposed by Watson and Crick this distribution of parental atoms is called *semiconservative* [239].

The DNA is again heated to denature the strands, cooled to allow re-annealing and heated to 72°C to allow primer extension. In this second round of amplification the correctly sized single stranded product is produced since the product from the first round is now a template and complementary DNA cannot be synthesized beyond the end of that DNA strand. The correctly sized fragment increases in quantity exponentially, with the final number of “correctly sized” double stranded molecules being approximately $2^n$ where $n$ is the number of cycles [240].
The Acquired Immunodeficiency Syndrome

**Historical Perspective**

The Acquired Immunodeficiency Syndrome (AIDS) first came to general medical attention in 1981 when investigators in Los Angeles [241] and New York [242] reported 2 unusual occurrences. The first was the occurrence of 5 cases of *Pneumocystis carinii* pneumonia reported in previously healthy young homosexual men in the United States in Los Angeles. This was highly unusual since Pneumocystis pneumonia occurs strictly in immunodeficient individuals. Such immunodeficiency is usually due to primary immunodeficiency diseases, secondary to diseases that have associated immunodeficiency or to immunosuppressive therapy [243]. At the same time a small epidemic of Kaposi’s sarcoma was reported from New York [244]. This was unusual because Kaposi’s sarcoma is a disease that occurred primarily in elderly men of Italian or eastern European Jewish ancestry or in those receiving immunosuppressive therapy, most often following renal transplantation [243]. These patients were believed to be suffering from a new syndrome of acquired cellular immune deficiency. It was named the Acquired Immunodeficiency Syndrome (AIDS) in 1982 and an AIDS case definition for surveillance was developed by the Centres for Disease Control.

**The AIDS case Definition**

The AIDS case definition is as follows,
"A case of a disease at least moderately predictive of a defect in cell-mediated immunity occurring in a person with no known cause for diminished resistance to disease" (Table 1)

Absence of all known underlying causes of cellular immunodeficiency (other than HTLV-III/LAV infection) and absence of all other causes of reduced resistance reported to be associated with at least one of those opportunistic diseases.

Although the initial reports of this condition concerned homosexual men [241, 242], it soon became apparent that the disease involved other groups, such as intravenous drug abusers [246], Haitian immigrants to the United States [247] [248] [249, 250], and haemophiliacs [251] [252].

The WHO established a clinical case definition in 1986 for use in countries where sophisticated equipment for CD4+ counts and the diagnosis of the indicator diseases doesn’t exist [253]. This requires the presence of two major and one minor sign in the absence of known immunosuppression (Table 2). Kaposi’s sarcoma and cryptococcal meningitis are considered AIDS defining in themselves.
Table 1: Diseases at least moderately indicative of underlying cellular immunodeficiency

(1993 CDC Revised Surveillance Definition of AIDS) [254]

<table>
<thead>
<tr>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiple or recurrent bacterial infections c</td>
</tr>
<tr>
<td>Candidiasis of the trachea, bronchi or lungs a</td>
</tr>
<tr>
<td>Candidiasis of the oesophagus b</td>
</tr>
<tr>
<td>Invasive cervical cancer d</td>
</tr>
<tr>
<td>Coccioidiomycesis, disseminated or extrapulmonary c</td>
</tr>
<tr>
<td>Cryptococcosis, causing central nervous system or other extrapulmonary diseases a</td>
</tr>
<tr>
<td>Cryptosporidiosis, intestinal causing diarrhoea for more than 1 month a</td>
</tr>
<tr>
<td>Cytomegalovirus disease (other than liver, lymph node or spleen) onset at age &gt; 1 month a</td>
</tr>
<tr>
<td>Cytomegalovirus retinitis (with loss of vision) a,b</td>
</tr>
<tr>
<td>HIV encephalopathy c</td>
</tr>
<tr>
<td>Chronic herpes simplex ulcer (&gt; 1 month duration) or pneumonitis or oesophagitis onset at &gt; 1 month age a</td>
</tr>
<tr>
<td>Histoplasmosis, disseminated or extrapulmonary a</td>
</tr>
<tr>
<td>Isosporiasis, chronic intestinal (&gt; 1 month duration) c</td>
</tr>
<tr>
<td>Kaposi’s sarcoma a,b</td>
</tr>
<tr>
<td>Lymphoid interstitial pneumonitis a,b</td>
</tr>
<tr>
<td>Lymphoma, primary brain a</td>
</tr>
<tr>
<td>Lymphoma (Burkitt’s or immunoblastic sarcoma) a</td>
</tr>
<tr>
<td>Mycobacterium avium complex or M. kansasii, disseminated or extrapulmonary a</td>
</tr>
<tr>
<td>Mycobacterium tuberculosis or acid-fast bacilli infection (species not identified), disseminated or extra-pulmonary c or pulmonary d</td>
</tr>
<tr>
<td>Pneumonia recurrent d</td>
</tr>
<tr>
<td>Pneumocystis carinii pneumonia a,b</td>
</tr>
<tr>
<td>Progressive multifocal leucoencephalopathy a</td>
</tr>
<tr>
<td>Toxoplasmosis of the brain, onset at age &gt; 1 month a,b</td>
</tr>
<tr>
<td>Wasting syndrome caused by HIV c</td>
</tr>
</tbody>
</table>

a If indicator disease is diagnosed definitively (e.g. by biopsy, culture) and there is no other cause of immunodeficiency, laboratory documentation of HIV infection not required.

b The presumptive diagnosis of indicator disease is accepted, if there is laboratory evidence of HIV infection (1997 addition)

c Requires laboratory evidence of HIV infection (1987 addition)

d Requires laboratory evidence of HIV infection (1993 addition; relates to both adults and adolescents)
Table 2 World Health Organization clinical case definition for AIDS

<table>
<thead>
<tr>
<th>Major Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight loss (&gt; 10% of body weight)</td>
</tr>
<tr>
<td>Chronic Diarrhoea (&gt;1 month)</td>
</tr>
<tr>
<td>Prolonged Fever (&gt;1 month)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Minor Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Persistent cough (&gt;1 month)</td>
</tr>
<tr>
<td>Generalised pruritic dermatitis</td>
</tr>
<tr>
<td>Recurrent Herpes Zoster</td>
</tr>
<tr>
<td>Oropharyngeal candidiasis</td>
</tr>
<tr>
<td>Chronic progressive and disseminated herpes virus infection</td>
</tr>
<tr>
<td>Generalised lymphadenopathy</td>
</tr>
</tbody>
</table>

*a* Adapted from the WHO weekly epidemiological record, 61: 69-73 (1986)
HIV Disease Classification and Staging

The most widely used classification of HIV disease is the 1986 CDC classification [255] (Table 3).

Table 3 1986 classification System for HIV infection

<table>
<thead>
<tr>
<th>Group</th>
<th>Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Acute infection</td>
</tr>
<tr>
<td>II</td>
<td>Asymptomatic infection</td>
</tr>
<tr>
<td>III</td>
<td>Persistent generalised lymphadenopathy</td>
</tr>
<tr>
<td>IV</td>
<td>Other Disease</td>
</tr>
<tr>
<td>A</td>
<td>Constitutional Disease</td>
</tr>
<tr>
<td>B</td>
<td>Neurological Disease</td>
</tr>
<tr>
<td>C</td>
<td>Secondary infectious diseases</td>
</tr>
<tr>
<td>C1</td>
<td>Specified secondary infections Listed in the CDC surveillance for AIDS b</td>
</tr>
<tr>
<td>C2</td>
<td>Other specified secondary infectious diseases</td>
</tr>
<tr>
<td>D</td>
<td>Secondary cancers</td>
</tr>
<tr>
<td>E</td>
<td>Other conditions</td>
</tr>
</tbody>
</table>

a Patients in Groups I and II may be sub-classified on the basis of laboratory evaluation

b Includes those patients whose clinical presentation fulfils the 1987 definition of AIDS used by the CDC for national reporting

The CDC classification system for HIV infection revised in 1993 defines three CD4+ T-lymphocytes and three categories of clinical disease (Table 4)
### Table 4 1993 revised classification system for HIV infection and expanded AIDS surveillance case definition for adolescents and adults

<table>
<thead>
<tr>
<th>CD 4+ T-cell Categories (Counts/mm³)</th>
<th>Clinical Categories</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A, asymptomatic</td>
</tr>
<tr>
<td></td>
<td>Acute (primary) HIV or PGL (^{b})</td>
</tr>
<tr>
<td>(1) &gt; 500</td>
<td>A1</td>
</tr>
<tr>
<td>(2) 200-499</td>
<td>A2</td>
</tr>
<tr>
<td>(3) &lt; 200</td>
<td>A3</td>
</tr>
<tr>
<td></td>
<td>B, symptomatic not A or C conditions (^{c})</td>
</tr>
<tr>
<td></td>
<td>B1</td>
</tr>
<tr>
<td></td>
<td>B2</td>
</tr>
<tr>
<td></td>
<td>B3</td>
</tr>
<tr>
<td></td>
<td>C, AIDS indicator conditions (^{d})</td>
</tr>
<tr>
<td></td>
<td>C1</td>
</tr>
<tr>
<td></td>
<td>C2</td>
</tr>
<tr>
<td></td>
<td>C3</td>
</tr>
</tbody>
</table>

\(^{a}\) Categories A3, B3, C1, C2 and C3 represent the expanded AIDS surveillance case definition. Persons with AIDS-indicator conditions (category C) as well as those with CD4+ cell counts < 200 cells/mm³ (categories A3 or B3) become reportable as AIDS cases in the U.S.A. and territories as from Jan 1\(^{st}\) 1993.

\(^{b}\) PGL, persistent generalised lymphadenopathy. Clinical category A includes acute (primary) HIV infection.


\(^{d}\) see Table A
Immunology of HIV infection

The HIV virus preferentially infects cells of the T-helper/inducer subset [256]. The receptor for the virus is the CD4+ cell surface antigen on the helper/inducer T cell subset [257]. The envelope glycoprotein gp120 of the HIV binds avidly to cell-surface CD4+ molecules and initiates fusion with the cell, involving gp41, and infection. Helper T-cells are the major target for infection, but as both macrophages and microglial cells also express CD4+ surface molecules, they too are susceptible to infection. Direct infection by the virus of follicular dendritic cells without CD4+ receptors can occur, the infected cells permit replication of the virus and can reinfect T-cells in vitro. Follicular dendritic cells are a major reservoir of HIV in AIDS. Once inside the cell the viral reverse transcriptase converts the viral RNA into DNA which then becomes integrated into the host cell genomic DNA where it can remain latent for long periods.

Infection of CD4+ cells leads to their destruction and an eventual drop in their numbers in both peripheral blood and lymphoid tissues. Since this cell is central to a variety of immunological reactions their depletion leads to profound cellular immunodeficiency, the hallmark of this disease. The depression of cellular immunity leads to a susceptibility to opportunistic infections and tumours that are the clinical manifestations of this disease [243]. There are many immunological consequences of HIV infection, amongst which are markedly diminished delayed hypersensitivity skin test responses. Production of γ interferon is also decreased [258] thereby causing reduced macrophage chemotaxis, and monocyte numbers may also be depleted [259, 260]. In addition neutrophil chemotaxis can also be diminished [261]. T-cell suppressor/cytotoxic cell numbers may also fall with progressive disease [243].
Polyclonal activation of B cells \[^{262}\] leads to elevated levels of immunoglobulins, most commonly IgG, IgD and IgA \[^{263}\]. Antibody responses to primary and secondary immunization may also be depressed \[^{264}\] \[^{265}\]. Elevated levels of circulating immune complexes are also found \[^{266}\]. Both the lymphocyte-mediated cytotoxicity of virally infected cells is diminished, as is natural killer cell (NK) cytotoxicity.

**Natural History of the Disease**

The most striking effect of infection with the HIV virus is the profound drop in CD4+ T-cells \[^{241}\]. In many the primary infection goes unnoticed, but some 40% present with an acute mononucleosis type picture \[^{254},^{267-269}\]. Symptoms consisting of fever, malaise, upper respiratory tract symptoms, myalgia and arthralgia, often with a transient maculopapular rash that lasts 2-3 weeks. Seroconversion occurs 8 to 12 weeks after the presumed exposure \[^{243}\]. Antibodies to p24 and envelope proteins gp 120 and gp 41 develop and cytotoxic T-cells curtail the viraemia with sequestration of the virus in lymphoid tissue \[^{58}\]. Primary infection is followed by a stage of so-called clinical latency, during which HIV replication and destruction of the immune system continues relentlessly in the lymphoid organs. Trapping of the viral particles complexed with antibody and complement stimulates follicular hyperplasia and infection of the follicular dendritic cells (FDC). In effect the follicles become the principal site of viral replication and infection of other cells of the immune system. Eventually follicular involution leads to an increase in viral burden and replication in peripheral blood mononuclear cells.

Early and middle stages of HIV infection begin 6 months after the primary infection and end with the appearance of AIDS defining illness. During this period the CD4+ count drops from normal levels to around 200-cells/μl. Most people are reasonably
well at this stage, although episodes of herpes zoster, thrush, skin infections and bacterial infections of the airways may occur. Neurological disease may precede the development of AIDS in infected individuals [243].

Up to 25% of infected individuals may have a lymphadenopathy syndrome. This consists of chronic generalised lymphadenopathy with or without constitutional symptoms such as low grade fevers, fatigue, malaise, night sweats, weight loss, thrush or diarrhoea. Up to 30% of people with this syndrome develop AIDS in a 2-to 3-year follow up [270, 271]. The development of thrush appears to be a poor prognostic sign, with about 60% of patients with it developing AIDS within a year [272].

Acute encephalopathy, acute and chronic meningitis, dementia, and myelopathy [273-275] are all associated with viral infection of the central nervous system. Isolation of the virus from both the cerebrospinal fluid and brains of patients with AIDS and neurological symptoms [273, 276, 277] and detection of IgG to the virus in the CSF of such patients has been documented [278]. In advanced HIV or AIDS the CD4+ cell count falls below 200 cells/μl and is characterised by opportunistic infections and tumours, and severe neurological disease.

**HIV/AIDS and the Eye**

The eye is one of the many organ systems that can be affected by diseases associated with AIDS. There is still no definitive evidence that HIV infection of ocular tissue leads to clinically important pathological effects [279], however sub clinical infection of retinal neural and endothelial cells has been reported [280]. A broad spectrum of disorders involve the eye, the most serious of which can result in blindness. The
ocular manifestations of AIDS can be grouped into four major categories (Table 5)

[127], to which three others can be added [281];

1. Cutaneous hypersensitivity reactions
2. HIV in the eye
3. Ocular manifestations of HIV infection in children
Table 5 Ocular disorders associated with AIDS

<table>
<thead>
<tr>
<th>Ocular disorders in AIDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. HIV-related retinopathy</td>
</tr>
<tr>
<td>2. Ocular infectious diseases (some of which are non-opportunistic, but with different characteristics in the immunocompromised)</td>
</tr>
<tr>
<td>a) Pathogens of the retina and choroid</td>
</tr>
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<td>i) cytomegalovirus</td>
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<td>ii) Mycobacterium avium-intracellulare</td>
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<td>iii) Cryptococcus neoformans</td>
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<td>iv) Toxoplasma gondii</td>
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<td>v) Herpes simplex virus</td>
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<td>ix) Candida albicans</td>
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<td>x) Histoplasma capsulatum</td>
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<td>b) Pathogens of the cornea and ocular adnexa</td>
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<td>i) Cytomegalovirus</td>
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<td>c) Papilloedema</td>
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HIV retinopathy

First described in 1982 [282], the most prevalent ocular complication of AIDS is a non-infectious microvasculopathy, the most common feature of which are cotton wool spots [283], which may also be associated with intraretinal haemorrhages [284].

Ocular Infectious Disease

Opportunistic infections of the eye are the most serious of the ophthalmic manifestations of AIDS. A variety of pathogens have been reported to cause intraocular infections in patients with AIDS, including, cytomegalovirus [125, 126, 140, 285, 286], Pneumocystis carinii [137, 287], Mycobacterium intracellulare [125, 140], Cryptococcus neoformans [125, 288, 289], Toxoplasma gondii [112, 126, 130], herpes simplex virus [125, 290, 291], herpes varicella zoster virus [285, 292-297], Candida albicans [126], and Histoplasma capsulatum [298]. When ocular infections do occur they are reliable indicators of invasive infections elsewhere in the body [125]. In a series of 35 AIDS patients reported by Pepose et al [125], ocular infections were found in 14 patients. In each patient, the pathogen infecting the eye was found in other tissues at autopsy. Ocular infections may produce the first symptoms of disseminated infections, and all patients who develop ophthalmic disease should evaluated systemically. There is a frequent association between ocular and intracranial disease [127, 284, 285]

Non-opportunistic infections associated with HIV infection

Certain infectious diseases will take on different characteristics or will occur with greater frequency in the HIV seropositive patient.
**Herpes zoster**

The correlation between severe herpes zoster in young adults and HIV seropositivity is well established [293, 299]. Herpes zoster ophthalmicus is a manifestation of both AIDS and ARC [293, 300], and may be the initial clinical manifestation of HIV infection [293]. Ophthalmic signs include vesicular lesions of the eyelids and eyelid margins, conjunctivitis, keratitis, and iridocyclitis. The anterior uveitis tends to be severe and prolonged and may recur [127]. Herpes zoster ophthalmicus in patients under 40 years of age is strongly associated with HIV positivity. During a 2 year follow-up 21% of these patients developed AIDS [300].

Both Varicella zoster [301-303] and herpes simplex virus [304, 305] are recognised as the causative agents of acute retinal necrosis. Acute retinal necrosis after herpes zoster ophthalmicus has been described in 17% of patients with HIV infection in the Western population [306]. The classically described triad of the ARN syndrome consists of (1) an arteritis and phlebitis of the retinal and choroidal vasculature, (2) a confluent, necrotizing retinitis that preferentially affects the peripheral retina, and (3) a moderate to severe vitritis. The progressive outer retinal necrosis (PORN) is a variant of necrotising herpetic retinopathy in patients with AIDS [307]. It has emerged as the second most common infection of the eye in AIDS, although it is still relatively uncommon [295]. It is caused by varicella zoster infection of the retina [308, 309], and is associated with a known history of cutaneous zoster in 67% cases [310]. Anterior chamber and vitreous inflammatory reactions are usually absent or minimal [308]. The typical lesions of PORN are characterised by multifocal creamy homogenous opacification of the deep retina scattered throughout the periphery, although the macula may be involved early in the disease [307, 308, 310, 311]. Although the disease is at first multifocal, lesions rapidly coalesce to form large areas of
retinal opacification. The course of the disease is characterised by early perivascular clearing of retinal opacification \(^{[307]}\). The condition was first described by Forster who noted the initial sparing of the inner retina and retinal vasculature, its relentless nature and poor response to acyclovir, and the development of retinal detachment \(^{[312]}\). Use of combination therapy would appear to be more effective at controlling, or at least slowing the progression of this condition \(^{[294, 313, 314]}\). These different forms of zoster retinitis (ARN as opposed to PORN) may reflect the patients immune function \(^{[307]}\). It is important to differentiate PORN syndrome from the more common CMV retinitis in HIV positive patients. Compared to PORN CMV retinitis progresses relatively slowly. The visual loss associated with CMV is related to the size and location of the individual lesions, whereas in PORN there is widespread retinal dysfunction. PORN is multifocal, in CMV disease there tends to be only two or three lesions in the eye. CMV is associated with haemorrhage, and the lesions are perivascular. CD4+ counts tend to be higher in patients with PORN \(^{[310]}\). Whilst both conditions are associated with retinal detachment it is a more common complication of PORN \(^{[307]}\).

**Herpes Simplex**

The incidence of dendritic keratitis, secondary to herpes simplex virus infection, is no greater than in the HIV negative population \(^{[315, 316]}\), but recurrence is more frequent \(^{[315]}\). Lesions tend to be peripheral and appear to be resistant to treatment \(^{[290]}\).

**Syphilitic uveitis**

Syphilitic uveitis is uncommon, occurring in less than 1% of the HIV population \(^{[317]}\) but CNS disease is commonly associated with ocular disease \(^{[318-321]}\). Syphilis serology should be performed on all patients with HIV and intraocular inflammation
The manifestations of ocular syphilis in HIV-infected hosts are protean; iridocyclitis, papillitis, optic perineuritis, retrobulbar neuritis, branch vein occlusion, neuroretinitis, chorioretinitis, intermediate uveitis, retinitis, panuveitis, periphlebitis, serous retinal detachment and vitritis have all been described [319, 321-326]. Anterior uveitis is the most common ocular manifestation, usually associated with panuveitis [317]. Syphilitic chorioretinitis in AIDS patients may have many features in common with CMV retinitis [327]. Lesions respond to penicillin therapy [127].

Opportunistic Infections

Cytomegalovirus retinitis

Cytomegalovirus (CMV) infection of the retina is by far the most common opportunistic infection of the eye [125, 282]. Prior to the advent of “Highly Active Anti-retroviral Therapy”, (HAART), CMV retinitis was estimated to occur in as many as 26-46% patients with AIDS [56, 125, 126, 140, 284]. It was the AIDS defining illness in 1.8% to 3% of patients [284, 328-330] with a lifetime risk of 44.9% [331]. Due to different levels of exposure to CMV between the various groups at risk for AIDS the incidence of CMV retinitis may be higher in homosexual and bisexual men [283].

CMV retinitis is a marker of advanced immunodeficiency, occurring in patients when CD4+ counts are low usually below 50 cells/μl [332-334].

Ophthalmoscopic appearance of CMV retinitis

Invasion of retinal cells following CMV viraemia results in a necrotising infection, leading to full thickness destruction of the retina [127]. Slowly progressive, centrifugally spreading, retinal opacification, which typically follows the blood vessels, is characteristic [335]. The disease process is frequently associated with haemorrhage and retinal vasculitis; vascular sheathing may be seen within or
adjacent to foci of infection. Lesions have irregular feathered borders and characteristically develop at the posterior pole adjacent to the major arcades. Occasionally multifocal, lesions can also develop in the retinal periphery. Progression occurs in one of two ways; new lesions may form away from pre-existing ones, probably by haematological spread, and more commonly previously uninvolved retina becomes infected by the lesions spreading at their borders.

Prior to the advent of HAART the condition was bilateral at presentation in 35% of cases [328], developed bilaterally in up to 80% of cases [336], and could develop in the contralateral eye whilst the patient was being treated with I.V. ganciclovir [335]. Resolution of the disease leaves retinal scarring and atrophy with mottling of the retinal pigment epithelium. Classically the retinitis is described as being associated with quiet media, although fine anterior keratic precipitates may be occasionally observed.

Visual loss occurs in CMV retinitis in a number of ways; widespread retinitis causes retinal atrophy and an absolute scotoma. Both the optic nerve and macula may be directly involved in the disease. Serous and rhegmatogenous retinal detachment may develop [127, 279].

Cystoid macular oedema, which occasionally responds to steroids, [337], also occurs [338-340]. The use of corticosteroids in the context of inactive CMV retinitis can be difficult as they have been shown to cause reactivation of cytomegalovirus [341]. Oral acetazolamide and topical non-steroidal agents have limited effectiveness [338].

*Manifestations of CMV retinitis in patients on highly active anti-retroviral therapy*

Since the use of anti-retroviral therapy, CMV retinitis has also been associated with vitritis [337, 342, 343], that may well reflect an improved immune response to the virus.
Vitritis may occur during inactive or active CMV retinitis [342], and has also been associated with optic nerve head neovascularisation [344].

**Cryptococcal Infection**

The prevalence of cryptococcal disease in different series of patients with AIDS in the United States ranges from 2% to 9% [345, 346]. *Cryptococcus neoformans* is the most common life-threatening fungal pathogen that infects AIDS patients [281, 288]. The most frequent manifestation is meningitis [347]. After the human immunodeficiency virus and *Toxoplasma gondii* it is the third most common infectious agent that causes neurological disease in patients with AIDS in Western countries [345]. Although rare [125, 289, 298], choroidal lesions can be the initial signs in disseminated cryptococcosis [348] and may coexist with CMV retinitis [349, 350].

The combination of low grade papilloedema, and headaches in an HIV positive patient, even in the absence of neck stiffness and fever, should alert the ophthalmologist to the possibility of cryptococcal meningitis.

**Infections with Candida albicans**

Despite the high incidence of mucocutaneous candidiasis in AIDS patients, both intraocular and intracranial *Candida* infections are uncommon [127], [197]. Intravenous drug abuse and prolonged catheterization are known risk factors for the development of candidaemia and *Candida* chorioretinitis [351, 352] which has been reported in an AIDS patient who was also an intravenous drug abuser [126].

As in the case of choroiditis due to *Cryptococcus, Candida* choroiditis is more common at autopsy than is diagnosed and reflects widespread dissemination of the organism [350].
Neoplasia

Kaposi’s sarcoma, B-cell lymphoma and squamous cell carcinoma are the most common malignancies reported in association with HIV infection.

Kaposi's Sarcoma

Kaposi’s sarcoma (KS), a malignant tumour, probably of vascular endothelial or lymphatic endothelial cell origin, [353, 354] may develop on the eyelids, eyelid margins, conjunctiva, most commonly in the inferior fornix [127], and rarely within the orbit [125, 140, 285, 286, 355, 356]. The incidence, reported as occurring in 24% of AIDS patients, [357] is declining [358, 359]. It is more common in homosexual and bisexual men [360]. Twenty percent of patients with systemic KS have ocular involvement [361], which may be the initial, but also the only manifestation of the disease [361]. Conjunctival lesions have been reported to occur in between 7-18% of AIDS patients with KS [140, 361]. Human herpesvirus type 8 (HHV-8) is postulated to be important in the causal pathway of KS [359].

Tumours are both chemo-sensitive and radiosensitive [127].

Lymphoma

Intraocular non-Hodgkin’s lymphoma (NHL) associated with AIDS is well recognised and was first reported in 1991 [362]. The phenotypes are usually B-cell and non-B-non-T-cell type lymphomas [363, 364] and include Burkitt lymphoma [347]. Affected patients are uniformly young adult males with a history of homosexuality and/or I.V. drug abuse [363, 365]. The tumours originate predominantly in extranodal locations. There is a strong predilection for the central nervous system [363]. It is unclear as to whether the incidence is changing [359, 366], but it would seem that HAART is having less effect on NHL than on other AIDS related malignancies [367]. The Epstein-Barr Virus, Human Herpes Virus type 8, HTLV-1 and hepatitis C
viruses have all been implicated aetiologically [368, 369]. Orbital lymphoma is rare [365, 370-373] and may occur simultaneously with intraocular disease [374]. Hodgkin’s lymphoma is seen rarely in association with HIV disease [363].

**Squamous cell carcinoma of the conjunctiva**

Aggressive squamous cell carcinoma may affect the conjunctiva and also the eyelid in HIV positive patients [375]. There is speculation that HIV allows the oncogenic potential of the Human papilloma virus (HPV) to be expressed [281]. Specimens positive for immunohistochemical staining have also been found to be positive for HPV DNA by in situ hybridisation, and by the polymerase chain reaction [376]. Conversely tests for HPV were negative in Malawian patients with conjunctival squamous cell carcinomas [377].

**Neuro-ophthalmological Manifestations.**

Neuro-ophthalmic abnormalities, including cranial nerve palsies, optic nerve disease, retrobulbar neuritis, visual field defects, pupillary abnormalities, cortical blindness, [125, 133, 197, 284-286] occur in AIDS. Most of these disorders result from infectious diseases of the central nervous system, most commonly cryptococcal meningitis, central nervous system toxoplasmosis, neurosyphilis [133, 197, 284-286], progressive multifocal leukoencephalopathy, and AIDS-related subacute encephalitis or intracranial tumours. Isolated, unilateral seventh nerve palsies are almost always associated with aseptic meningitis [197].

**HIV in the eye**

In 1985, the HIV virus was first isolated from human tears [378, 379]. Subsequently, the virus has been isolated from conjunctival [380] and corneal epithelium [381], which is probably infected by the tears [382], from aqueous [382], the retina [280, 383], iris [383] and vitreous [384]. Concern has developed that the presence of HIV in ocular
tissue and tears could lead to disease transmission via corneal transplantation \[^{385}\] although no such case has ever been reported \[^{254}\]. The Eye Bank association of America has adopted two screening techniques to detect corneal donors infected with HIV \[^{386}\]. First there is the recognition of high risk individuals according to a profile developed by the Centres for Disease Control and secondly the determination of seropositivity using the ELISA test. In the United States the seroprevalence amongst donors is in the range of 0.33% to 0.68% \[^{254}\].

**Ocular manifestations of HIV infection in children**

Infection from mother to child accounts for the large majority of HIV infection in infants and children \[^{254}\]. The vertical route is a relatively efficient mode of transmission as compared to that through sexual contact \[^{254}\]. In industrialised countries the range of vertical transmission is from 15% to 25%, \[^{387}\]. The severe degree of B-cell dysfunction in children with perinatal HIV infection represents a striking difference between adults and children in the clinical manifestations of HIV infection \[^{254,265}\]. This immunological abnormality explains the severe and recurrent bacterial infections in such children \[^{388}\]. The rate of disease progression is much greater than in adults \[^{254}\].

From the available data it seems that the ocular aspects also differ in adults and children \[^{389-391}\]. Cotton-wool spots are rare in children \[^{391}\], and may only be found in older children who acquire the infection through transfusion of blood or blood products \[^{389}\].

When CMV retinitis occurs in children it is often bilateral, with advanced disease typically being present in one eye at the time of diagnosis as young children rarely complain of unilateral visual loss. Treatment of CMV retinitis in children is based upon the adult regimens. Response to treatment is comparable for the induction
dose, but children seem to experience earlier recurrences on the maintenance therapy [254]. A recent report on a limited number of cases concluded that combined intravenous ganciclovir and foscarnet for induction and maintenance offered improved long-term control of CMV retinitis in children [392].

An increased incidence of optic atrophy reported may in fact have been related to maternal drug and alcohol abuse [393]. An increased incidence of strabismus in children with a symptomatic HIV infection has also been reported [389].

ddI toxicity in children causes a peripheral retinopathy that appears after 9 months and is non-progressive upon cessation of the drug [389].

To date the literature regarding the PCR amplification of *T. gondii* DNA from aqueous samples remains contradictory. Detection of *T. gondii* DNA following PCR amplification of aqueous samples from patients with active retinochoroiditis seems highly variable. No consensus appears to exist as to the best methodology. A variety of different genes have been used as the primer targets, and both nested and single PCR reactions with a variety of different detection methods have been used. It is unclear whether additional DNA extraction steps are required to facilitate this process and hence maximise the sensitivity of the reaction.

This thesis considers these issues. The gene that facilitates the maximum sensitivity and subsequent detection of *T. gondii* DNA was determined, PCR inhibition by aqueous fluid was investigated, and in addition the issue of whether an additional DNA extraction step would facilitate subsequent PCR amplification was specifically addressed. Having optimised these parameters the amplification of DNA from aqueous samples from patients with active *T. gondii* retinochoroiditis is then investigated.
In addition, this thesis looks at aspects of HIV disease in patients in both this country and in Uganda. Following the introduction of highly active anti-retroviral therapy, survival of patients with HIV disease has changed. The change in survival of patients with CMV retinitis following the introduction of highly active anti-retroviral therapy is investigated.

The greatest burden of HIV disease falls on the medical carers in the developing world. The majority of published work in this field concerns urban populations and tertiary referral hospitals. A study has been undertaken to evaluate the workload of rural eye care programmes in this field.
Chapter 2: Can Toxoplasma gondii DNA be detected in ocular fluids by PCR from patients with active retinochoroiditis?

Background

Toxoplasma retinochoroiditis is usually a clinical diagnosis based upon examination of the retina and recognition of the characteristic lesion. Diagnosis can be difficult in atypical cases, as might occur in the elderly, the immunocompromised, including AIDS patients, or in those cases where adequate fundoscopy is precluded by overlying vitreous opacity.

Since the clinical picture is often that of a panuveitis, associated with marked inflammatory activity in the anterior chamber, and as anterior chamber taps are associated with fewer complications than sampling from the vitreous cavity, an investigation was undertaken to determine if T. gondii DNA could be detected in aqueous humour. The development of a highly sensitive polymerase chain reaction (PCR) protocol to identify T. gondii DNA will help in the early diagnosis of unusual retinochoroiditis and facilitate the institution of appropriate treatment.

The use of the polymerase chain reaction to amplify and subsequently detect DNA within microorganisms in a range of tissues, and particularly those which are difficult to culture [394] or for which sample volumes are small has proved extremely valuable [395].

In this study we have directly compared the sensitivity of previously described primer sequences [229, 396, 397] in the amplification of specific target sequences from the B1, P30, and ribosomal T. gondii genes in both water and aqueous humor and have developed a PCR based protocol that is capable of detecting a single T. gondii
tachyzoite. Secondly we have investigated the most efficacious method of DNA extraction and we have applied these techniques to a variety of ocular samples to assess the diagnostic value of PCR amplification of *T. gondii* DNA from aqueous humour, vitreous and retinal samples obtained from patients with active retinochoroiditis.

**Morphological variants of retinal Toxoplasma lesions**

Three morphological variants of retinal *Toxoplasma* have been described \[^{67}\],

(1) large destructive lesions;

(2) inner punctate lesions;

(3) punctate outer (or deep) retinal lesions

**Large destructive lesions:**

This lesion appears to be the most common and most ominous lesion since it produced prolonged disease associated with visual loss \[^{67}\]. Active retinitis is dense, yellowish white and elevated and involves an area usually larger than the optic nerve head. An intense inflammatory reaction produces marked oedema around the lesion. Dense vitritis may prevent direct visualization of both the disc and the lesion. Marked anterior uveitis is common. The lesion becomes gray as disease activity diminishes, with pigmentation developing at the edge. Vitreous clearing may take months. Cataract, cystoid macular oedema and tractional retinal detachment are all associated with this type of lesion.

**Punctate inner retinal lesions:**

These lesions, with a shorter duration of disease activity than large destructive lesions are the second most frequent variant \[^{67}\]. Associated with minimal oedema
and overlying vitreous activity, such lesions are either single or multifocal, small, gray areas of active retinitis which may occur both centrally and peripherally.

**Deep (outer retinal) punctate lesions:**
Initially described by Gass [398], and later by Friedman [67], these multifocal lesions are always located at the macula or near the optic nerve and are deep punctate lesions with subretinal exudate. They may resemble the disciform lesion associated with ocular histoplasmosis [67]. Differential diagnosis includes acute posterior multifocal placoid pigment epitheliopathy, serpiginous choroiditis [63], and diffuse unilateral subacute neuroretinitis [399]. The active retinitis in this variant occurs at the level of the outer retina and RPE and clinically is usually seen as a patch of gray retina with few vitreal inflammatory cells [399].

**Toxoplasmic Papillitis**
Occasionally, a foci of inflammation within or directly adjacent to the optic nerve head may develop [63]. Severe unilateral papillitis is associated with vitritis, nerve fibre-bundle defects and no apparent retinal foci [400]. If papillitis is the only lesion diagnosis maybe difficult. Diagnosis is facilitated if a focus of inflammation adjacent to the optic nerve head is associated with an old *Toxoplasma* scar [401]. The differential diagnosis of *Toxoplasma* papillitis includes optic neuritis, anterior ischaemic optic neuropathy, sarcoidosis, optic disc vasculitis [402], tumours of the optic nerve head, and other causes of retinitis that may have an associated optic nerve involvement [63].
Neuroretinitis

Neuroretinitis causing acute, unilateral visual loss has been reported in juveniles or young adults. Characteristically optic nerve oedema and a stellate distribution of hard exudates at the macula associated with an overlying vitritis is described. Afferent pupillary defect, anterior chamber activity, and peripheral retinochoroidal scars may occur. Other features that help to differentiate this condition from idiopathic neuroretinitis (which maybe associated with a history of preceding viral illness), are positive toxoplasma serology, recurrence, and on occasion permanent loss of vision. Both of these conditions may be associated with retrobulbar pain.

Involvement of the Anterior Segment

Toxoplasma retinochoroiditis is often associated with either granulomatous or non-granulomatous anterior uveitis. Redness of the external eye, cells and protein in the anterior chamber, large “mutton fat” keratic precipitates, and posterior synechiae are characteristic. Iris nodules and occasionally iris neovascularisation occur. Although Toxoplasma has been demonstrated in the iris of numerous domestic animals, proof of the existence of the parasites in human anterior uvea has never been obtained. For this reason, anterior uveitis alone, without a coexisting fundal lesion, should not be considered a manifestation of toxoplasmosis. Granulomatous anterior uveitis allegedly due to toxoplasmosis has been described, however the eye was so severely damaged that others argued the case was one of a pan-uveitis rather than a pure iridocyclitis. Since actual infection of the anterior segment has never been proven in the immunocompetent host, the uveitis is assumed to be a hypersensitivity reaction.
Other Complications of Ocular Toxoplasmosis

Other complications of ocular toxoplasmosis include chronic iridocyclitis [67], cataract formation, secondary glaucoma [68], band keratopathy [67, 406], retinal detachment [67, 144] and optic atrophy secondary to optic nerve involvement.

Visual loss associated with Ocular Toxoplasmosis

Direct involvement of the fovea by retinochoroiditis is the most important sequela of ocular toxoplasmosis [31]. In sixty cases of ocular toxoplasmosis 12% had active retinitis within 5 degrees of the umbo, and a further 12% demonstrated some evidence of macular oedema [407].

Choroidal neovascularisation may occur as a late complication of the disease [408, 409] with new vessels located either directly at the border of the scar, or at a distance with feeder vessels arising from the scar [31]. Retinochoroidal anastomoses have also been observed to occur in toxoplasmosis [31], with one study placing the incidence at 2.7% [410].

Vision may be compromised by the overlying vitritis alone, but macular oedema is often observed in the acute or subacute phases of the disease [69], and can even occur when the macula is away from the focus of retinitis in a phenomenon similar to that seen in pars planitis [31].

Visual Prognosis

Friedmann and Knox reported that 41% of their patients with toxoplastic retinochoroiditis ultimately suffered permanent unilateral visual loss of 20/100 or less. In 88% cases this severe visual loss was due to macular involvement with a
lesion, the rest were due to large destructive lesions in the periphery. Visual loss was independent of the number of preceding episodes of active retinitis but was associated with duration of the active episode. Mild to moderate visual loss (20/40 to 20/70) was seen in an additional 16% cases [67].

**Differential Diagnosis of Infectious Retinitis**

A number of other infectious diseases remain important causes of retinitis. Furthermore, because specific antimicrobial therapy can be curative and prevent long-term visual sequelae, early diagnosis of infectious causes of uveitis is an important aspect of these conditions.

**Tuberculosis**

Ocular involvement occurs in approximately 1-2 % cases of patients with TB [411]. Tuberculosis can involve both the anterior and posterior segments of the eye as well as the orbit and adnexae [412]. In miliary TB, tubercles can be seen in the choroid, giving the impression of unifocal or multifocal choroiditis [412]. Eales’ disease, a disorder associated with retinal vasculitis and vitreous haemorrhage is also associated with TB [412]. A number of both infectious and non-infectious conditions may cause ocular disease similar to TB. For example, the retinal periphlebitis that results from tuberculosis can mimic the ocular disease associated with both sarcoidosis and syphilis. Underlying immunosuppression, such as AIDS should always be considered in patients with ocular TB [412].

**Syphilis**

There has been a substantial increase in primary and secondary syphilis, and an increased incidence of congenital syphilis, especially in the inner city [413, 414]. Syphilis is also a problem in patients coinfectcd with HIV. This group of patients
may not mount a serological response to the treponemal infection and thus elude diagnosis \[4^{12}\]. Standard therapy may be insufficient to eradicate the infection in the immunocompromised \[4^{15}, 4^{16}\].

**Congenital Syphilis**

Overt or subclinical multifocal areas of choriocapillaris inflammation may occur in congenital syphilis. Post-inflammatory changes include variable chorioretinal atrophy, especially peripherally; retinal pigment epithelial proliferation; narrowed retinal blood vessels and epiretinal gliosis. Syphilis remains the leading apparent cause for childhood chorioretinitis \[4^{17}\]. A sprinkling of pigmented and depigmented spots (salt and pepper fundus) can appear in the periphery, selectively involve the posterior pole, or appear in isolated quadrants \[4^{17}\].

**Acquired Syphilis**

In acquired syphilis similar changes appear, although the posterior pole and juxtapapillary areas are more likely to be affected. Changes are bilateral in about 50% cases, with yellow grey foci of choroidal infiltrates which later become confluent and which are frequently associated with overlying retinal oedema, vitreous cells and disc oedema \[4^{18}\]. Pigment clumping and migration tends to occur at the borders of the areas of choroiditis and along the retinal blood vessels. Residual changes include fine peripheral pigmentation, focal or scattered chorioretinal atrophic scars, and sometimes preretinal fibrosis \[4^{17}\]. Chronic exudative retinal detachment with uveal effusion has also been reported \[4^{19}\].

The eye can be affected at any stage in syphilis. Both retinochoroiditis and neuroretinitis, without choroidal involvement almost always occurs in secondary syphilis \[4^{20}, 4^{21}\], and are frequently associated with meningitis \[4^{21}, 4^{22}\]. Other features may include vitritis, disc oedema, exudative retinal detachment, and
perivasculitis. Both retinochoroiditis and neuroretinitis can rarely be associated with tertiary meningovascular syphilis [422-424]. Features include vasculitis, venous and arterial occlusive disease, macular oedema, exudative retinal detachment, vitritis, pseudoretinitis pigmentosa, and chorioretinal neovascular membranes. Syphilis can also cause a necrotising retinitis [425, 426].

**Lyme Disease**

The tick born parasite *Borrelia burgdorferi* is the cause of the disease Lyme disease, which classically is described as having three stages. Early infection is divided into stage 1 (local) and stage 2 (disseminated). Late infection is categorised as stage 3 (persistent). The disease can manifest ocular lesions, the commonest being conjunctivitis reported as occurring in 11% of people suffering from stage 1 disease [427]. Other ocular findings reported in the early stages of Lyme disease include retinal vasculitis [428] [429], and panophthalmitis has been reported in which spirochetes were found in vitreous specimens [430]. Meningeal symptoms can occur with diffuse choroiditis, cystoid macular oedema, and exudative retinal detachments [431]. Lyme disease can cause an intermediate uveitis [432, 433], neuroretinitis and optic neuritis [412]

**Fungal Disease**

In general, fungal infection of the choroid, retina and vitreous occurs in post operative patients, immunosuppressed patients with mycotic infections, immunocompetent patients with systemic mycotic infections, intravenous drug abusers, and in patients with ocular trauma. Occasionally a patient without a known risk factor may develop a fungal endophthalmitis.
Fungal endophthalmitis usually presents as a chronic panuveitis. Fluffy deep yellow-white retinal or choroidal lesions are frequently present, and the vitreous frequently contains “puff-balls”.

*Candida Species*

*Candida albicans* is the most frequent cause of fungal endophthalmitis [434], and usually occurs in chronically ill patients with an indwelling catheter [351], but is also a frequent complication of intravenous drug abuse [435].

The diagnosis of *Candida* endophthalmitis is usually straightforward in a chronically ill patient with positive blood cultures. The usual assumption is that the same organism in the blood is responsible for the ocular disease. Nevertheless multiple organisms may present in the blood and eye of severely immunocompromised patients. In addition some intravenous drug abusers who develop a candidal infection from emboli do not develop a systemic infection. These patients frequently develop multiple yellow-white deep retinal or choroidal lesions associated with vitreous puff balls and retinal haemorrhage [412].

*Aspergillus Species*

Aspergillosis is the second most common disseminated fungal infection [434]. It has been reported as the cause of endophthalmitis in both immunosuppressed patients and in intravenous drug abusers [436, 437]. *Aspergillus* infection of the eye presents as a vitritis with puff-balls, and yellow white lesions in the retina similar to those seen in *Candida* endophthalmitis. Also similar to candidal infections redness, pain, hypopyon, retinal haemorrhages and decreased vision can occur. *Aspergillus* may be difficult to culture from blood [438], and may occur in the absence of evidence of systemic infection [437].
Endogenous fungal endophthalmitis

Endogenous fungal endophthalmitis can occur from a variety of organisms including Coccidioides immitis, Cryptococcus neoformans, Histoplasma capsulatum, Sporotrichum schenckii and Blastomyces dermatitidis. Such lesions have been described as necrotising retinitis or retinochoroiditis and been misdiagnosed as ocular toxoplasmosis. These infections are all less common than diseases caused by either Candida or Aspergillus infection and represent a diagnostic challenge to the clinician. Nocardia asteroides can cause disease of similar appearance to fungal ocular infections.

The use of PCR in the diagnosis of ocular toxoplasmosis

PCR amplification of sequences within the B1, P30 and ribosomal genes of T. gondii have been assessed in a variety of ocular tissues, in both the immunocompetent and immunocompromised patient.

Several groups have reported the use of PCR analysis of aqueous humor in the diagnosis of ocular toxoplasmosis using oligonucleotide primers for DNA sequences within the B1 gene, the P30 gene, and the ribosomal gene with highly variable results. Others have reported the use of PCR on both vitreous and retina samples.

P30 Protein

Four major tachyzoite proteins have been identified. The most abundant surface protein, P30, constitutes 3%-5% of the total tachyzoite protein. It is an integral membrane protein of the tachyzoite, with a molecular weight of 30,000 under reducing conditions. P30 is a stage specific antigen; it is detected only in
the rapidly dividing and invasive tachyzoites and is not expressed by either the bradyzoite of tissue cysts, or sporozoites of the oocyst stage of the parasite \[457\]. This abundant antigen is homogeneously distributed on the surface of both the extracellular and intracellular tachyzoites \[454, 458\]. P30 also appears to be a major component of the parasitophorous vacuole \[49\], and may play a role in the parasite's ability to invade the host cell \[459\]. Sequencing of the gene coding for the P30 protein shows it to be a single copy gene \[456\] that is highly conserved in different strains tested to date \[40, 460\]. The protein is highly immunogenic, the serum from virtually all people exposed to *T. gondii* containing a high titre of antibodies directed against it \[456\]. In humans it induces the production of IgG \[455\], IgM \[461\] and IgA \[461\] antibodies. This response to P30 is seen in congenital, acute and chronic human serum \[456\]. Purified P30 has been successfully tested as a single antigen for the serodiagnosis of acute and chronic toxoplasmosis \[462\]. P30 stimulates peripheral blood mononucleocytes from seropositive individuals to secrete IFN-γ \[463\], one of the major mediators of resistance to *T. gondii* infection \[59\].

Monoclonal antibody to P30, in addition to binding to P30, also demonstrates in vitro parasiticidal activity in the presence of human complement. P30 is the major radioiodinated tachyzoite antigen identified by human and mouse antitoxoplasma antisera \[457\]. Convalescent human serum has been found to contain high titres of anti-P30 antibody. Mice immunised with affinity column purified P30 protein produced a dose-dependent antigen-specific IgG and IgM response, however, when challenged with tachyzoites, immunised mice showed a statistically significant increase in mortality over non-immunised control mice. In addition immunised mice had an increased number of intra cerebral cysts compared to the control mice \[457\]. This is in contrast to other investigators who found immunisation with P30 in
combination with liposomes conferred high levels of protection against *T. gondii* [460].

**Excreted/secreted antigens**

ESA are proteins derived from organelles called "dense granules" which are distributed throughout the cytoplasm of the tachyzoite [17]. These antigens are reported to consist of 90% of the Toxoplasma antigen circulating in the host [464, 465]. ESA are highly immunogenic in eliciting both antibody [466, 467] and T-cell mediated responses [468]. Of the ESA studied, p28 and p23 have been the most widely studied. P28 is an intracellular antigen [469, 470] present in the dense granules [471] and is secreted by the tachyzoites [470], and is also a minor component of the parasitophorous vacuole [470, 471]. The p23 protein is present in the dense granules of both tachyzoites and bradyzoites and is secreted into the reticulate network within the parasitophorous vacuole. This antigen is readily recognised by patient serum in chronic infection but less so by those with acute infection [472].
Materials and Methods

Unless otherwise stated, all chemicals used were purchased from Sigma Chemical (Poole, UK) and were of the highest grade available.

Preparation of *T. gondii* tachyzoites, inoculation of mice and isolation of DNA.

Infection free mice (MF1 strain) were inoculated intraperitoneally with 0.5ml of RH strain tachyzoites at a concentration of 500,000/µl from a cell line that was originally isolated from a human encephalopathy sample [39]. Tachyzoites isolated from peritoneal fluid were counted using a modified Fuchs Rosenthal CSF counting chamber. DNA was extracted following suspension in 2ml lysis buffer (50mM Tris-HCl pH 8.0, 0.1 M NaCl, 10mM EDTA, 2% sodium dodecyl sulphate and 0.2µg/ml Proteinase K) and incubation at 50°C for 3 hours. Samples were extracted with phenol: chloroform (1:1), chloroform, precipitated with ethanol in the presence of 300mM sodium acetate pH5.2, and air dried following washing with 70% ethanol. DNA pellets were resuspended in TE buffer (10mM Tris-HCl pH7.5, 1mM EDTA), and concentration assessed by both UV absorbance at 260 nm and by comparison with Hind III digested lambda DNA standard. The DNA was diluted to 10ng/µl with water and stored at -20°C. Serial dilutions of this DNA were then used as template for optimising PCR reactions.

Serial dilutions of tachyzoites

RH strain tachyzoites were obtained from The Public Health Laboratory Service at St. George's Hospital, Tooting, London, U.K. The tachyzoites were isolated from peritoneal fluid and counted using a modified Fuchs Rosenthal CSF counting chamber. A fresh solution of tachyzoites of known concentration of $4.6 \times 10^7$
cells/ml was used and serial ten fold dilutions were prepared in water. Identical 10 
µl aliquots of each dilution were then immediately processed for comparison of 
DNA extraction techniques. Results of PCR were compared in each case with direct 
PCR of extracted genomic *T. gondii* DNA in water starting with a concentration of 
1ng, and direct PCR dilutions of tachyzoites in water.

*Optimising polymerase chain reaction sensitivity of B1, P30 and Ribosomal Primers*

Using the reaction conditions for previously described primer sequences [229, 396, 397] 
as a basis, the following reaction parameters were assessed across a range of values 
in order to maximize the reaction sensitivity for the detection of known amounts of 
*T.gondii* DNA in 1µl of sterile water in a total of 25µl PCR volumes

A) Annealing temperature
B) Cycle numbers
C) dNTP concentration
D) Magnesium concentration
E) Primer concentration and,
F) Taq concentration

*Collection of aqueous fluid*

The extraocular environment was sterilised with 5% Povidone Iodine solution prior 
to surgery. Approximately 100-200µl of aqueous fluid was withdrawn using a 27G 
(0.33mm) needle via a limbal paracentesis prior to routine cataract surgery in 
patients with no evidence on fundoscopy of *T.gondii* scars.

In order also to test the effect, if any, of PCR amplification in the presence of 
inflamed aqueous, 150µl of aqueous was also obtained from a patient with post- 
operative fibrinous uveitis.
**Collection of Vitreous Fluid**

Vitreous sampling was undertaken under aseptic conditions through the pars plana at the time of standard three port pars plana vitrectomy and 200-400μl of vitreous was aspirated. A vitreous cutter was inserted through the pars plana 3mm behind the limbus in aphakic eyes and 4mm behind the limbus in phakic eyes.

Prior to sampling both aqueous and vitreous, informed consent was obtained from all patients. The institutional review board at Moorfields Eye Hospital approved the protocol for collection of aqueous samples. Ethical committee approval was also obtained at Sao Paolo. This research followed the tenets of the Declaration of Helsinki at all times.

**Retina**

The sample of retinal tissue from an enucleation sample was obtained from the Department of Pathology at the Institute of Ophthalmology, Bath Street, London, U.K. Microscopy had confirmed the presence of *T.gondii* cysts within the tissue sections.

**Sample preparation from Paraffin embedded tissue**

Retinal samples containing tissue cysts were deparaffinised according to a technique described by Wright and Manos [473]. Briefly, 15μm sections of paraffin embedded retina were cut, transferred to a 1.5-ml microcentrifuge tube, deparaffinised by two xylene extractions. One ml of xylene was added to each tube, the closed tubes were then mixed at room temperature for about 30 minutes. Tissue and residual paraffin were then pelleted by centrifugation at 14,000 rpm for 5 minutes. Following a second xylene extraction the xylene was removed by pipette. The samples were then washed twice with 100% ethanol to remove the organic solvent. 0.5ml of 100%
ethanol was added to each tube the contents of which were then mixed by inverting. Samples were then centrifuged for 5 minutes at 14,000 rpm and the ethanol removed by pipette and the process repeated. The samples were resuspended in 100μl digestion buffer (50mM KCl, 10mM Tris-HCl pH 8.3, 1mM EDTA, 2.5mM MgCl₂, 0.1 mg/ml gelatin, 0.45% octylphenol-ethylene oxide condensate, 0.45% polyoxyethylene sorbitan monolaurate, 200μg/ml proteinase K) and were digested for 3 hours at 55°C. Proteinase K was inactivated, by heating for nine minutes at 95°C. After 3 minute centrifugation at 14,000 rpm, 10μl of supernatant was used for PCR amplification. 1 in 2 and 1 in 10 dilutions of this material also underwent PCR amplification. In addition 10ng of genomic T. gondii DNA in 10μl sterile water was also subjected to both the deparaffinising process and the digestion process so as to serve as positive controls for these techniques. Negative extraction controls consisted of sterile water that had undergone both the deparaffinising and digestion processes. Outer PCR positive controls consisted of 1 ng of genomic T. gondii DNA in 10μl sterile water that underwent PCR without prior extraction or digestion, and a final PCR negative control consisted of sterile water that had not been subjected to either extraction or digestion.

Additional positive and negative nested reaction controls consisted firstly of 1 ng of genomic T. gondii DNA in 10μl water that had not been subjected to first round PCR and secondly 10μl sterile water that had not been subjected to first round PCR.

**Nested PCR protocols**

One μl of first round product was used as template for subsequent nested amplification. Both the negative control sample from the first round amplification as
well as a second round negative control of sterile water only was included in the nested amplification.

**Amplification of the Bl gene:**

Two pairs of oligonucleotide primers directed against the Bl gene of *T. gondii* [229] were used to perform a nested PCR reaction using purified *T. gondii* DNA as a template (Table 9).

**Bl gene: First round amplification:**

PCR reactions contained 10mM Tris-HCl, pH8.3 (at 25°C), 50mM KCl, 2mM MgCl$_2$, 0.1μM each primer, 0.1mM each dNTP, 1.25U Taq DNA polymerase and varying quantities of purified *T. gondii* DNA. Reactions were cycled forty times with denaturation at 93°C for 10 seconds followed by annealing at 57°C for 10 seconds and finally an extension step at 72°C for 30 seconds. PCR negative control sample omitted template DNA which was substituted with sterile water.

**Bl gene: Nested amplification.**

Nested reactions contained 1μl first round product, 10mM Tris-HCl, pH8.3 (at 25°C), 50mM KCl, 3mM MgCl$_2$, 0.5μM each primer, 0.1mM each dNTP, and 1U Taq DNA polymerase. Nested PCR reactions were cycled forty times using a denaturation step of 93°C for 10 seconds, followed by annealing at 62.5°C for 10 seconds and extension at 72°C for 15 seconds. Negative control samples from first round amplification as well as an additional second round negative control of sterile water were included in the nested reactions.
Nested Primer sets identical to those used by Savva et al. [396] and based on the published sequence of the T. gondii P30 gene [456] were used to amplify regions of the P30 gene using a nested PCR approach (Table 10).

**P30 gene; first round amplification.**

Each 25μl PCR reaction mix consisted of 10mM Tris-HCl, pH8.3 (at 25°C), 50mM KCl, 2mM MgCl₂, 0.2μM of each primer, 0.1mM of each dNTP, and 0.5U of Taq DNA polymerase and varying concentrations of purified T. gondii DNA. Reactions were cycled 35 times using a denaturation step of 95°C for 1 minute, annealing at 65°C for 1 minute and extension at 74°C for 3 minutes. PCR negative control sample omitted template DNA which was substituted with sterile water.

**P30 gene: nested amplification.**

1μl of product was used as template for the nested amplification. Each 25μl reaction contained 10mM Tris-HCl, pH 8.3 (at 25°C), 50mM KCl, 1mM MgCl₂, 0.4μM of each primer, 0.1mM of each dNTP, and 0.5U of Taq DNA polymerase. Nested reactions consisted of 35 cycles using a denaturation step at 95°C for 1 minute, followed by annealing at 66°C for 1 minute and extension at 74°C for 3 minutes. Negative control samples from first round amplifications as well as an additional second round negative control of sterile water were included in nested PCR reactions.

**Ribosomal gene amplification**

Oligonucleotide primers used to amplify ribosomal DNA (Table 11) were based on those used by Cazenave et al [397], and were based on the published sequence of the small subunit ribosomal RNA gene of T. gondii [474]. PCR reactions (25μl) consisted of 10mM Tris-HCl, pH8.3 (at 25°C), 50mM KCl, 2.5mM MgCl₂, 0.2μM of each
primer, 25μM of each dNTP, and 1.5U Taq DNA polymerase and varying concentrations of purified *T. gondii* DNA. Reactions were heated at 95°C for 10 minutes, and cycled 35 times using a denaturation step of 95°C for 10 seconds, followed by annealing at 60°C for 30 seconds, and extension at 74°C for 1 minute. PCR negative control sample omitted template DNA which was substituted with sterile water.

**PCR amplification of bacterial and fungal genomic DNA using *T. gondii* B1 gene primers.**

To show primer specificity, outer and nested B1 amplification reactions were carried out on 25μl reaction mixtures that contained 10ng of genomic DNA from a variety of fungal and bacterial species. DNA from the following species was used as template; *C. albicans, C. parapsilosis, C. tropicalis, A. fumigatus, S. aureus, S. epidermidis, S. pneumoniae, S. viridans, P. aeruginosa, S. marcescens, E. coli, P. mirabilis, K. pneumoniae*. Positive control consisted of 1ng genomic *T. gondii* DNA. The outer negative control consisted of the 25μl PCR reaction without added DNA.

**Amplification of Human lymphocyte genomic DNA using *T. gondii* B1 gene specific primers.**

To ascertain that no human sequences were amplified with B1 specific primers and that the presence of human lymphocyte DNA did not inhibit detection of *T. gondii* B1 template, 1ng of *T. gondii* genomic DNA was added to different amounts of human DNA in sterile water and amplified using B1 gene primers.
Amplification of the B1 gene in inflamed aqueous.

To establish the volume of inflamed aqueous responsible for inhibition of B1 gene sequence, 1ng of *T. gondii* genomic DNA was added to various volumes of inflamed aqueous and subjected to PCR amplification. To establish that the B1 PCR reaction retained its sensitivity even in the presence of inflamed aqueous, various quantities of *T. gondii* genomic DNA were added to 5μl volumes of aqueous that had been obtained from a patient with post-operative fibrinous uveitis. Samples were subjected to direct PCR analysis without prior DNA extraction.
Comparison of DNA extraction methods using serial dilutions of tachyzoites in water

Following determination of the sensitivity and confirmation of the specificity of the primers directed against the B1 gene, it was determined whether DNA extraction prior to PCR further enhanced the sensitivity of this reaction. Serial dilutions of tachyzoites (see previous section) were subjected to DNA extraction methods. Ten μl of each dilution sample, along with tachyzoite dilutions that had not been subjected to DNA extraction then underwent nested PCR amplification.

Table 6: DNA extraction techniques used prior to PCR amplification of B1 gene.

<table>
<thead>
<tr>
<th>DNA extraction Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heating at 95°C for 5 minutes</td>
</tr>
<tr>
<td>Heating at 95°C for 10 minutes</td>
</tr>
<tr>
<td>Heating at 95°C for 20 minutes</td>
</tr>
<tr>
<td>Sonication for 5 minutes</td>
</tr>
<tr>
<td>Sonication for 10 minutes</td>
</tr>
<tr>
<td>Sonication for 20 minutes</td>
</tr>
<tr>
<td>Cellular lysis and Protein Digest</td>
</tr>
<tr>
<td>Cellular lysis and Protein Digest followed by phenol: chloroform extraction</td>
</tr>
<tr>
<td>2M NH₄OH</td>
</tr>
</tbody>
</table>

B1 gene: First round amplification

10 μl of each of the tachyzoite dilutions underwent first round amplification in a total PCR volume of 25μl. PCR reactions contained 10mM Tris-HCl, pH 8.3 (at 25°C), 50mM KCl, 2mM MgCl₂, 0.1μM each primer, 0.1mM each dNTP, 1.25U
Taq DNA polymerase and varying quantities of purified *T. gondii* DNA. Reactions were cycled forty times with denaturation at 93°C for 10 seconds followed by annealing at 57°C for 10 seconds and finally an extension step at 72°C for 30 seconds. PCR negative control sample omitted template DNA which was substituted with sterile water.

**B1 gene: Nested amplification.**

Nested reactions contained 1µl first round product, 10mM Tris-HCl, pH8.3 (at 25°C), 50mM KCl, 3mM MgCl₂, 0.5µM each primer, 0.1mM each dNTP, and 1U Taq DNA polymerase. Nested PCR reactions were cycled forty times using a denaturation step of 93°C for 10 seconds, followed by annealing at 62.5°C for 10 seconds and extension at 72°C for 15 seconds.

**Patient samples**

Nineteen samples from patients with active *T. gondii* retinochoroiditis were collected, consisting of 17 aqueous and 2 vitreous samples. In addition a retinal sample containing *T. gondii* tissue cysts was also subjected to PCR amplification.

Seventeen consecutive patients referred to either Moorfields Eye Hospital, London, England, or to the Department of Ophthalmology, Laboratory of Transplant Immunology, University of Sao Paulo, Sao Paulo, Brazil were enrolled in this study and underwent anterior chamber paracentesis. All patients had lesions that were clinically compatible with a diagnosis of active Toxoplasma retinochoroiditis at the time of paracentesis with aqueous activity ranged from 1+ cells to 3+ cells [475]. None of the patients were on anti-Toxoplasma treatment prior to sampling. All patients had concurrent vitreous activity. None of the patients were known to have risk factors for HIV infection. Aqueous sampling took place from 1 day to 6 weeks
from the onset of symptoms. A variety of presenting symptoms were noted, including blurred vision, floaters, pain and red eyes. In 10 of the cases this was their first known episode of active ocular toxoplasmosis. The remainder had had between 1 and 6 known previous episodes of active retinochoroiditis. Patients in whom the diagnosis was in any doubt were excluded.

Two vitreous samples, taken at the time of rhegmatogenous retinal detachment repair by standard three-port pars plana vitrectomy underwent PCR analysis. Both patients had developed retinal detachment during episodes of active retinochoroiditis.

As previous publications have indicated that greater than 20% of aqueous or vitreous fluid can inhibit PCR reactions [395, 476] we routinely used 5μl samples of aqueous or vitreous per 25μl PCR reaction for subsequent work. Initially from each patient sample four 5μl aliquots were taken. To three of these patient samples either 10ng, 10pg or 100fg of *T. gondii* DNA was added to test the inhibitory capacity of these fluids. No *T. gondii* DNA was added to the final aliquot. All aliquots were then heated to 95°C for 20 minutes prior to undergoing PCR amplification using primers directed against the B1 gene. In the presence of a negative PCR result the amount of PCR inhibition was investigated for each sample by diluting the ocular sample 1in 2, 1in 5, 1in 10 and 1in 20 and repeating the PCR amplification.

Genomic DNA from retinal sections was isolated as previously described [473].

**Polymerase chain reaction**

Once it had been established which of the above DNA extraction techniques enabled maximum sensitivity as judged by the subsequent B1 gene nested PCR amplification
of *T. gondii* DNA from aqueous samples spiked with tachyzoites, all samples from patients with *Toxoplasma* retinochoroiditis were subjected to the same regimen.

**Visualisation and confirmation of PCR amplification products**

10μl B1 and P30 amplification products were visualised under UV illumination following electrophoresis on 1-2% TBE/agarose gels and staining with ethidium bromide. 20μl of ribosomal gene amplification products were visualised on 4% TBE/Metaphor agarose gels (Flowgen Instruments, Lichfield, UK) or on 8-10% TBE/polyacrylamide gels. (Biorad Laboratories, Hemel Hempstead, UK). A molecular weight marker was included in each run (1.0-kb ladder, Cat. no. 15615-016; Gibco BRL, Paisley, Scotland, or 1-kb ladder, Cat. no. G571A; Promega UK Ltd., Southampton, UK)

**DNA Sequencing of PCR products**

Prior to sequencing of PCR products, amplified DNA from PCR reactions was purified using the Geneclean II kit (BIO 101 Inc. California, USA). PCR products were excised from agarose/TBE gels, solubilised in sodium iodide and recovered into solution according to the manufacturer’s instructions. PCR fragments were directly cycle sequenced in both directions (using the outer most primers for each gene) on an ABI prism automated DNA sequencer (model 377 version 2.1.1). DNA sequences were compared with target sequences and found to be identical in all cases.
Results

Amplification of \( B1 \) gene from \( T. gondii \) DNA.

A single amplicon with a predicted size of 193 base pairs was amplified using the first round \( B1 \) gene primers. This reaction was capable of detecting 50 fg of \( T. gondii \) DNA following analysis on ethidium bromide stained TBE/agarose gels (Figure 2-1). It has been estimated that this is the amount of DNA contained within a single \( T.gondii \) organism [17]. When 1\( \mu \)l of first round product was used as template in a nested amplification, a 96 base pair target sequence was amplified, but showed no increase in the level of detection of \( T. gondii \) DNA (50 fg). Following the nested reaction, however, there was a significant increase in the product yield (Figure 2-2). The nested reaction did not yield product from either first or the second round negative controls. Both first round and nested PCR products were cycle sequenced which confirmed the amplified products were identical to the published sequence of the \( T.gondii \) B1 gene [229].

\( B1 \) gene: Amplification of \( T. gondii \) DNA in aqueous.

First round amplification of \( T. gondii \) DNA in 20% aqueous (\( T. gondii \) DNA spiked into 5\( \mu \)l aqueous in a total reaction volume of 25\( \mu \)l) was able to detect 1 pg of \( T. gondii \) DNA (Figure 2-3: ). Following second round amplification using the nested primers this protocol was effective in detecting 50 fg of \( T. gondii \) DNA (Figure 2-4).

Amplification of \( T. gondii \) P30 gene.

Amplification of the P30 gene using outer primers resulted in a single product of approximately 914 base pairs. Amplification of the P30 gene using this protocol was able to detect 1 pg of purified \( T.gondii \) DNA following analysis on ethidium bromide
stained TBE/ agarose gels (Figure 2-5). When 1µl of first round PCR product was used as template in the nested amplification, a single amplicon of approximately 520 base pairs was observed. The sensitivity of the reaction was 50fg of *T.gondii* DNA (Figure 2-6). Both the outer and nested products were subject to cycle sequencing and confirmed products were identical to published sequences.

*Amplification of the P30 gene from *T. gondii* DNA in aqueous.*

First round amplification of *T. gondii* DNA in 20% aqueous (*T. gondii* DNA spiked into 5µl aqueous in a total reaction volume of 25µl) was able to detect 1ng of *T. gondii* DNA (Figure 2-7) Second round amplification using the nested primers was, however, only able to detect 1pg *T.gondii* DNA in the presence of 20% aqueous humour compared to 50fg *T.gondii* DNA in the absence of aqueous humour (Figure 2-8).

*Amplification of 18S rDNA gene from *T. gondii* DNA.*

An 88 base pair sequence from the 18S rDNA was used as the target sequence for amplification. Using oligonucleotide primers based on those used by Cazenave *et al.* amplification of the ribosomal gene from *T.gondii* allowed detection of 1pg *T. gondii* DNA (Figure 2-9). Sequence analysis confirmed the identity of the 88 bp amplicon.

*Amplification of 18S rDNA gene from *T. gondii* DNA in aqueous.*

When different concentrations of purified *T. gondii* DNA was supplemented with 5 µl aqueous, ribosomal gene amplification detected only 1pg of purified *T. gondii* DNA (Figure 2-10).
B1-gene specific primers do not amplify sequences from human, bacterial or fungal genomic DNA.

Primers directed against the *T. gondii* B1 gene did not produce an amplicon when the template genomic DNA was derived from human lymphocytes, (Lane 7, Figure 2-12), bacterial or fungal species.

Amplification of B1 gene from 1ng *T. gondii* DNA is not inhibited in the presence of human lymphocyte DNA.

The presence of increasing amounts human lymphocyte DNA (10-150ng) did not inhibit either the amplification of 193bp B1 PCR product from 1ng purified *T. gondii* genomic DNA in 20% normal human aqueous or the nested amplification of 96bp product generated from 193bp B1 PCR product (Figure 2-11 and Figure 2-12).

Amplification of the B1 gene in inflamed aqueous.

When 1ng of genomic *T. gondii* DNA was added to a first round PCR reaction containing inflamed aqueous from a patient with fibrinous uveitis, product inhibition was seen when the reaction mix contained greater than 20% inflamed aqueous (Figure 2-13). When 1 µl of the outer reaction was added to a final nested reaction mix of 25 µl a 96 base pair amplicon was produced even when the outer reaction contained 60% inflamed aqueous. A decrease in the amount of product however was seen in the nested reaction if the outer reaction contained more than 40% by volume (10µl) of inflamed aqueous (Figure 2-14). As previous publications have indicated that 20% of aqueous fluid per PCR reaction can inhibit the reaction [476] we have routinely used 5µl samples of aqueous per 25µl reaction for subsequent work. After adding varying amounts of genomic *T. gondii* DNA to 5 µl of inflamed aqueous a 193 bp amplicon was produced down to a sensitivity of 1pg *T. gondii* DNA (Figure 2-15). When 1µl of this outer reaction was added to a nested reaction in a final
volume of 25μl the reaction was capable of detecting 50 fg of *T. gondii* DNA which is an identical sensitivity to the detection of *T. gondii* DNA in water (Figure 2-16).

**Comparison of DNA extraction methods**

For all DNA extraction methods used, following first round amplification using B1 gene primers a single amplicon with a predicted size of 193 base pairs was amplified. When 1μl of first round product was used as template in a nested amplification, a 96 base pair target sequence was amplified. Products were visualised on ethidium bromide stained TBE/agarose gels

*Direct PCR of Sample*

First round amplification of sample demonstrated the sensitivity of the PCR reaction to be equal to DNA equivalent to 4.6 x 10³ tachyzoites (Figure 2-17). Nested amplification improved the level of detection to 4.6 tachyzoites (Figure 2-17).

*DNA extraction by heating to 95 °C for 5, 10., or 20 minutes*

PCR amplification of samples in which DNA was extracted by heating at 95°C for 5 minutes showed no improvement in levels of detection over direct PCR after first round amplification. However nested amplification showed this extraction procedure to improve the level of detection to less than a single tachyzoite. Amplification of DNA sequences from less than one tachyzoite is possible due to the fact that the B1 gene is a thirty five-fold repetitive gene sequence within the *T.gondii* genome. A 10 or 20 minute heating extraction further improved first round amplification sensitivity to a level of detection of 4.6 x 10³ and 46 tachyzoites respectively (Figure 2-17), and maintained the level of sensitivity to less than a single tachyzoite after nested amplification (Figure 2-17).
DNA extraction by sonication for 5, 10 or 20 minutes prior to PCR

Cellular lysis within samples by sonication for 5 minutes showed no improvement of sensitivity of subsequent first round PCR over direct PCR. However nested PCR amplification sensitivity remained at a level of detection of a single tachyzoite. Sensitivity of first round PCR was further enhanced by prior sonication for 10 or 20 minutes to a level of detection of $4.6 \times 10^2$ and 46 tachyzoites respectively. Nested amplification maintained the level of sensitivity to a single tachyzoite.

Cellular lysis, Protein Digestion and partial DNA extraction

DNA extraction by cellular lysis and protein digestion, a method modified from Aouizerate et al. [449], demonstrates a level of sensitivity of $4.6 \times 10^4$ after subsequent first round PCR amplification. This method of extraction was unable to improve the sensitivity of nested amplification over that of heating or sonicating the samples (Table 7). Additional phenol:chloroform extraction was unable to improve first round PCR sensitivity (Table 7). Phenol:chloroform extraction was also unable to improve the sensitivity of first round PCR.

Ammonium Hydroxide

Treatment of samples with ammonium hydroxide to cause cellular lysis was capable of detecting DNA to a level of $4.6 \times 10^4$ tachyzoites after first round PCR and 1 tachyzoite following nested amplification.

Summary of comparison of DNA extraction techniques

Comparison of DNA extraction techniques revealed that heating of samples for 20 minutes or sonication for 20 minutes to be the most efficacious based on their limit of detection of 46 tachyzoites after just one round of PCR. Direct PCR, appeared to have a limit of detection of 4.6 tachyzoites after nested PCR. All DNA extraction
techniques showed improved sensitivity with nested PCR and demonstrated a limit of detection of just one tachyzoite (Table 7).
Table 7 Table of DNA extraction after 1st round and Nested amplification of *T. gondii* DNA using primers directed against the B1 gene

<table>
<thead>
<tr>
<th>DNA extraction Method</th>
<th>Sensitivity after 1st round PCR amplification (number of tachyzoites)</th>
<th>Sensitivity after Nested PCR reaction (number of tachyzoites)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct PCR (no prior DNA extraction)</td>
<td>$4.6 \times 10^1$ tachyzoites</td>
<td>$4.6$ tachyzoites</td>
</tr>
<tr>
<td>Heating at 95°C for 5 minutes</td>
<td>$4.6 \times 10^1$ tachyzoites</td>
<td>1 tachyzoite</td>
</tr>
<tr>
<td>Heating at 95°C for 10 minutes</td>
<td>$4.6 \times 10^2$ tachyzoites</td>
<td>1 tachyzoite</td>
</tr>
<tr>
<td>Heating at 95°C for 20 minutes</td>
<td>$4.6$ tachyzoites</td>
<td>1 tachyzoite</td>
</tr>
<tr>
<td>Sonication for 5 minutes</td>
<td>$4.6 \times 10^1$ tachyzoites</td>
<td>1 tachyzoite</td>
</tr>
<tr>
<td>Sonication for 10 minutes</td>
<td>$4.6 \times 10^2$ tachyzoites</td>
<td>1 tachyzoite</td>
</tr>
<tr>
<td>Sonication for 20 minutes</td>
<td>$4.6$ tachyzoites</td>
<td>1 tachyzoite</td>
</tr>
<tr>
<td>Cellular lysis and Protein Digest</td>
<td>$4.6 \times 10^4$ tachyzoites</td>
<td>1 tachyzoite</td>
</tr>
<tr>
<td>Cellular lysis and Protein Digest followed by phenol: chloroform extraction</td>
<td>$4.6 \times 10^4$ tachyzoites</td>
<td>1 tachyzoite</td>
</tr>
<tr>
<td>2M NH$_4$OH</td>
<td>$4.6 \times 10^8$ tachyzoites</td>
<td>1 tachyzoite</td>
</tr>
</tbody>
</table>

**PCR analysis of patient samples**

Five microlitre aliquots of patient aqueous or vitreous samples were heated at 95°C for 20 minutes prior to being subjected to nested PCR reaction using primers directed against the Toxoplasma B1 gene. No product was visualised after both 1st round and subsequent nested amplification.

To see if such negative results were due to PCR inhibition by the patient samples, further 5μl aliquots from each of these first 11 patient samples were spiked with either 1ng, 1 pg or 100fg of *T. gondii* DNA. These samples along with another unspiked sample were then subjected to nested PCR, again using primers against the B1 gene, following DNA extraction by heating to 95°C for 20 minutes. The results
from this showed variable PCR inhibition between different samples (Table 8). Subsequently all patient samples were then subjected to PCR in 5μl neat samples and also 5μl samples diluted 1 in 2, 1 in 5, 1 in 10, and 1 in 20.
Table 8: Patient samples showing variable PCR inhibition when spiked with specific quantities of *T. gondii* DNA

<table>
<thead>
<tr>
<th>Aq/Vit</th>
<th>1st / 2nd round of PCR</th>
<th>5µl neat sample</th>
<th>unspiked sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1ng 1pg 100fg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pt1</td>
<td>1st - - -</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2nd ++ - -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pt2</td>
<td>1st ++ + -</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2nd ++ ++ -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pt3</td>
<td>1st - - -</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>2nd ++ - -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pt4</td>
<td>1st ++ + -</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2nd ++ ++ -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pt5</td>
<td>1st ++ - -</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>2nd ++ ++ -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pt6</td>
<td>1st ++ - -</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>2nd ++ ++ -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pt7</td>
<td>1st ++ - -</td>
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</tr>
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<td></td>
<td>2nd ++ ++ ++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pt8</td>
<td>1st ++ - -</td>
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<td></td>
<td>2nd ++ ++ ++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pt9</td>
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</tr>
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<td></td>
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<td></td>
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</tr>
<tr>
<td>Pt11</td>
<td>1st ++ - -</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>2nd ++ ++ ++</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Despite this both inner and nested PCR failed to produce an identifiable product on gel electrophoresis in any of the aqueous samples.

In the vitreous samples obtained from one of the patients with *T. gondii* retinochoroiditis a 193 bp was detected only in the positive control sample that had
been spiked with 1ng *T. gondii* genomic DNA after first round PCR amplification (Figure 2-18).

Nested amplification of 96bp B1 PCR product was detected in all of this patient sample (Figure 2-19).

No amplifiable product was detected on gel electrophoresis after PCR on the second vitreous sample.

*B1 gene amplification of T gondii DNA from paraffin embedded sections.*

Four deparaffinised retinal sections from a patient with histopathological evidence of *T. gondii* cysts, typical of Toxoplasma retinochoroiditis, were subjected to PCR using primers directed against the B1 gene. After first round amplification a single 193bp product was visualised from reactions in three of the four sections on 2% agarose gels (Figure 2-20). All negative controls remained negative. Positive and negative controls which had been subjected to the deparaffinising procedure all remained positive and negative respectively (Figure 2-21)

An identical product was visualised from all positive controls. After nested amplification, 96bp products were visualised from all retinal sections (Figure 2-22). Positive and negative controls which had been subjected to the deparaffinising procedure all remained positive and negative respectively after nested amplification (data not shown).
DISCUSSION

Molecular biological methods have been investigated to aid in the clinical management of *T. gondii* retinochoroiditis condition. The use of PCR to amplify and subsequently detect DNA within microorganisms in a range of tissues, and particularly those which are difficult to culture \[^{1}\] or for which sample volumes are small has proved extremely valuable \[^{2}\]. The ability to identify *T. gondii* DNA in ocular samples will provide direct evidence of the presence of the organism within the eye and therefore be helpful in determining the diagnosis of *T. gondii* infection and subsequent patient management. To this end we have looked at the PCR analysis of ocular fluid samples from patients with a clinical diagnosis compatible with ocular toxoplasmosis and discuss the possible contribution of PCR in the diagnosis of *T. gondii* retinochoroiditis. Further analysis of various DNA extraction techniques prior to PCR analysis of ocular fluids is presented.

Primer selection

The B1, P30 and ribosomal DNA genes are highly conserved in all *T. gondii* strains tested to date \[^{229, 233, 460}\], and both the B1 and ribosomal genes are multiple copy genes, within the *T. gondii* genome making them ideal targets for PCR amplification.

The B1 gene is a thirty five-fold repetitive gene sequence with unknown function. Within eukaryotes ribosomal DNA is frequently repeated, and within *T. gondii* there are over one hundred highly conserved copies within the genome \[^{233}\]. The P30 gene is expressed only by the tachyzoite \[^{457}\] and encodes for the most abundant surface protein, comprising 5% of the total tachyzoite protein \[^{454, 456}\].
Sensitivity

Nested amplification of both the P30 and B1 genes allowed detection of as little as 50fg of *T. gondii* DNA in water. In aqueous, only amplification of the B1 gene yielded this level of sensitivity. Nested amplification of the P30 gene in aqueous allowed detection only to a level of 1pg *T. gondii* DNA.

The ribosomal gene was the least sensitive of these reactions only allowing detection of the predicted 88bp target down to a DNA concentration of 1 pg in water. This is perhaps surprising given that it is the most highly repeated of the gene sequences studied but may be due at least in part to the fact that the ribosomal amplification protocol used only a single reaction \cite{397}. Nested reactions increased the yield of product for both the other genes amplified.

Inter-laboratory variability is a confounding factor when analysing results and trying to compare different methodologies used at different centres. This study has used protocols published by others and optimised all three PCR reactions in the one laboratory. We have concluded that, at least in our hands, the B1-gene primers and PCR protocol are the most sensitive of the three tested.

Selection of B1 gene primers

PCR amplification of DNA target sequences within the B1 gene had several advantages. Like the P30 it allowed for greater sensitivity than amplification of the ribosomal gene. However, it has been shown that the P30 primers are less specific than those of the B1 gene as they have been demonstrated to amplify DNA targets of both the *Nocardia* species \cite{477} and *M. tuberculosis* DNA \cite{478}. We have shown that the *T. gondii* B1 primers do not amplify DNA targets from either human DNA or a variety of bacterial and fungal species. In the latter reactions, the sensitivity remains
unchanged both in the presence of increasing amounts of human DNA, and in the presence of the increased levels of protein found in inflamed aqueous. It is, however, possible that other parasites present in the tissue may be amplified by these PCR techniques. In order to ensure the specificity of the PCR reaction primer sequences were compared with all other sequenced DNA available from current databases and were found to be non-complimentary to any other known sequence. The possibility does exist, however, that a related organism could be identified using these primers, an organism whose gene sequence remains hitherto unknown. In order to minimise this possibility the PCR reaction is performed under stringent conditions. Reaction specificity is facilitated by using the highest possible annealing temperatures compatible with maximum reaction sensitivity. Finally sequencing of all amplified PCR products was performed and confirmed the identity of the genes amplified.

**DNA extraction**

A number of biological fluids contain substances that inhibit PCR \(^{479-481}\). A recent multicentre collaborative study to determine the reliability and reproducibility of PCR in the detection of *M. tuberculosis* DNA in sputum, saliva and water \(^{482}\) and another one using *T. gondii* DNA added to amniotic fluid \(^{234}\) found highly variable sensitivity and specificity among the different laboratories involved. In each study the participants used the same target sequence for DNA amplification. However, DNA extraction, detection and amplification conditions were different among the centers, and no conclusions could be drawn regarding the optimal conditions for PCR.

Different methods for DNA extraction prior to PCR have been described. The classic method of DNA extraction involves the incubation of sample with proteinase K in the presence of sodium dodecyl sulphate (SDS) and subsequent phenol-
chloroform extraction with ethanol precipitate \[^{83}\]. Treatment with proteinase K in the presence of SDS lyses the cell and liberates the DNA tightly bound to chromatin. Proteins are then extracted with phenol and chloroform, and the nucleic acids then precipitated with ethanol.

SDS is inhibitory to *Taq* polymerase at concentrations as low as 0.01% \[^{484}\]. This effect can be abolished by the addition of a nonionic detergent such as Tween 20 or Laureth 12 \[^{485}\]. If the Proteinase K is subsequently heat denatured prior to PCR amplification, SDS/Proteinase K cellular lysis is compatible with direct PCR \[^{485}\] without phenol/ chloroform extraction and precipitation \[^{486-489}\]. Alternative simple DNA extraction procedures have been used. These include the use of silica beads \[^{490}\], procedures that involve the use of detergents \[^{491, 492}\], chaotropes, proteases, alkali wash and heat lysis \[^{493}\], sonication \[^{491, 494}\] or heat denaturation alone \[^{485, 495}\].

Using PCR for the amplification of *M. tuberculosis* DNA from clinical isolates, Afghani and Stutman \[^{496}\] found that a simple boiling method of DNA extraction was more sensitive and no less specific than a conventional chemical method using lysozyme and silica particles.

The use of PCR on ocular samples has been previously described, however, there appears to be no consensus as to whether PCR sensitivity is enhanced by the prior use of DNA extraction, and if so which is the most appropriate method to use in these circumstances.

Using aqueous, vitreous and sub-retinal fluid taken from HIV patients with a clinical diagnosis of CMV retinitis, Fox *et al* \[^{497}\] were able to amplify CMV DNA from all the samples tested. As much as 30μl of each specimen was used in a total PCR mixture of 100μl. Prior to the PCR, all samples had been boiled for 10 minutes in
order to denature proteins present in the specimens that might inhibit the PCR reaction. Conversely, Limpens and Kijlstra reported a potent PCR inhibitor in vitreous that could not be removed by boiling. Wiedbrauk et al described a PCR inhibitor in both normal aqueous and vitreous fluids. As little as 0.5µl of vitreous fluid and 20µl of aqueous fluid completely inhibited DNA amplification in a 100µl reaction mixture. Further they found that the inhibition was not primer specific, nor was it due to chelation of Mg$^{2+}$ ions or DNase activity of the ocular fluids. They concluded that whilst the specific nature of the inhibitory substance in ocular fluids had yet to be determined, it appeared to act directly on the Taq polymerase, and was found in much higher concentrations in vitreous than aqueous. Unlike Fox et al they found that the inhibitor was completely resistant to boiling, but that this inhibitory effect was completely removed by a single chloroform-isoamyl alcohol (24:1) extraction, or more simply by diluting the specimen. Previous work in this laboratory has demonstrated lack of PCR inhibition if the volume of vitreous does not exceed 20% of the final reaction volume.

We have compared a variety of sample preparations and assessed their effect on the ability to subsequently amplify *T. gondii* DNA by PCR in aqueous samples that had been previously spiked with known quantities of *T. gondii* tachyzoites. These extraction procedures were compared to samples that had not undergone any prior DNA extraction. The methods assessed include simple heating, heating the samples with ammonium hydroxide, sonication, and proteinase digestion both with and without phenol: chloroform extraction. We concluded that the use of DNA extraction on aqueous samples enhanced the sensitivity of the subsequent PCR reaction when compared to PCR that was performed on aqueous samples that had not undergone prior DNA extraction. However we found that by simply heating the
samples allowed for the detection of DNA from a single tachyzoite by nested PCR amplification, and by heating for 20 minutes allowed maximum sensitivity after first round amplification. We therefore conclude that the use of more complex DNA extraction techniques confer no advantage over this simple method. Purification of nucleic acids from specimens by classical procedures (involving detergent mediated lysis, proteinase treatment, extractions with organic solvents and ethanol precipitation) is tedious and time consuming, and when dealing with small samples, such as vitreous or aqueous, significant amounts of DNA can be lost \[^{485}\]. Furthermore, the many steps involved in the purification of nucleic acids from specimens increases the risk of transmission of DNA from sample to sample leading to false positive results \[^{490}\]. It has been argued, therefore, that such techniques are incompatible with diagnostic procedures \[^{485}\], and should therefore be avoided if simpler and equally efficacious methods are available.

**Application to clinical samples**

Initial investigations used spiked aqueous samples to assess the efficacy of PCR detection of *T.gondii*.

The technique of heat extraction at 95°C for 20 minutes followed by PCR amplification using primers directed against the B1 gene of *Toxoplasma* was then performed on 17 aqueous samples and 2 vitreous samples from patients with a clinical appearance consistent with that of active *T.gondii* retinochoroiditis and on samples of retinal tissue that had had tachyzoites identified within them on microscopy. Only one of the vitreous samples (Figure 2-19) and the retinal tissue (Figure 2-22) on which PCR examination was performed allowed detection of the predicted 96 base pair product when PCR products were analysed on agarose gels.
The vitreous sample had been obtained from a patient with suspected *T. gondii* retinochoroiditis. In this case a vitrectomy was performed as the patient had developed a giant retinal tear, but had an active lesion that was clinically suspicious of *T. gondii* retinochoroiditis and was already receiving prednisolone EC 20mg bd., and clindamycin 150mg bd. for ocular toxoplasmosis. A positive result was obtained with no ‘vitreous inhibition’ of the PCR reaction as all the vitreous samples spiked with genomic DNA from *T. gondii* were positive with a sensitivity of 100fg.

PCR products using these primers had previously been sequenced so as to ensure product specificity.

Several authors have reported the use of PCR for the identification of *Toxoplasma* DNA in aqueous [141, 231, 444-451, 499] with highly variable results.

Others have reported the use of vitreous [452], and retina [136, 445]. Brézin *et al* [446] using oligonucleotides directed against the B1 gene performed PCR and intraocular antibody analysis on aqueous samples taken from 17 patients with clinical presentations consistent with ocular toxoplasmosis. Only 3 of the cases had positive PCR results. Ocular inflammation had been present for at least 2 weeks prior to paracentesis. Garweg *et al* [447], again using PCR directed against the B1 gene found that only one of 43 aqueous samples contained detectable amounts of DNA. Aouizerate *et al* [500] using the ribosomal gene as target DNA were able to identify *Toxoplasma* DNA in 7 of 23 cases and concluded that the sensitivity of PCR was limited by the small volume of the aqueous samples. Moreover as Garweg pointed out [447] it is not possible to discount the presence of false positive results. Using aqueous samples taken from patients with AIDS and necrotising retinitis Verbraak *et al* [141] were only able to amplify *Toxoplasma* DNA in 1 of 5 cases with a final diagnosis of *T. gondii* retinochoroiditis, CMV DNA was also detected in the same
sample. In another case that also had a final diagnosis of ocular toxoplasmosis only CMV DNA could be detected. De Boer et al. [450] could only detect *Toxoplasma* DNA in 31% (4 of 13 aqueous or vitreous samples) from immunocompetent patients with active *T. gondii* retinochoroiditis. *T. gondii* DNA was not detected in either aqueous or vitreous from patients with AIDS and a clinical diagnosis of *T. gondii* retinochoroiditis (4 patients). In both of these latter two studies primers directed against the ribosomal gene were used.

We have found that primers directed against the B1 gene were consistently the most sensitive in the identification of *T. gondii* DNA in aqueous samples that had been spiked with known quantities of DNA. When patient samples were subjected to PCR amplification positive controls were used that showed the B1 PCR reaction being used was capable of identifying *Toxoplasma* DNA at a level compatible to the amount of DNA found in between 1 and 10 organisms. Thus the lack of positive results found in this series is not likely to be due to a lack of sensitivity of the PCR reaction used.

It could be argued that false negative results accounted for the lack of amplifiable DNA in this study. This is unlikely to be the case as all samples were subject to DNA extraction prior to being subjected to PCR and diluted as recommended by the study of Fox et al. Even in the presence of fibrinous uveitis, the protocol used was able to amplify *T. gondii* DNA. The inhibition of PCR by aqueous could be further reduced if nested as opposed to single PCR was used.

The advantages of aqueous sampling over vitreous sampling in terms of both comfort and safety for the patient makes this an attractive technique to use. The use of PCR–based assays for the diagnosis of cytomegalovirus (CMV), varicella zoster (VZV), and Herpes simplex virus (HSV) to amplify target DNA from vitreous
samples from patients with viral retinitis has been well documented [501-504]. De Boer et al [450] were able to amplify CMV DNA from ocular fluids from 21 of 23 patients with AIDS and active CMV retinitis. CMV DNA and VZV DNA were successfully amplified from vitreous samples taken from patients with viral retinitis and AIDS in a study by Knox, [505]. Herpes simplex DNA was also detected in 6 vitreous samples from 6 patients with necrotising retinitis. Successful amplification of CMV DNA from aqueous samples from HIV positive patients with CMV retinitis [141, 497, 506] and from sub retinal fluid [497] has also been carried out. Varicella zoster DNA has also been amplified from both aqueous and subretinal fluid from patients with acute retinal necrosis [507]. These PCR protocols have been of especial use where the aetiological agent has remained in doubt [505, 508] and may be a more sensitive method for confirming diagnosis in HIV patients than analysis of local antibody production [509]. However aqueous humour may not be as reliable as vitreous for the detection of DNA from aetiological agents causing retinitis [510].

The Toxoplasma gondii parasite has been observed in human aqueous using contrast phase microscopy by Habbegger [511] and has been isolated from aqueous in a case of severe Toxoplasma panuveitis [64]. Rehder et al [152] reported the finding of a Toxoplasma cyst in a biopsy obtained from the iris of a patient with AIDS. T.gondii organisms have been cultured from infected retinal tissue [512] and the organism isolated from subretinal fluid [222]. Toxoplasma antigen has been detected in vitreous in experimental ocular toxoplasmosis [204], however, actual infection of the anterior segment has never been demonstrated in an immunocompetent host [154].
Therefore the non-granulomatous or granulomatous type of uveitis that can occur in ocular toxoplasmosis \[^{153}\] is assumed to be a hypersensitivity phenomenon \[^{70}\].

Recently, Klaren \textit{et al.} \[^{80}\] have demonstrated that in cases of presumed chronic recurrent ocular toxoplasmosis, most ocular fluid samples contained antibodies that were specific for a 28-kD antigen. They also showed that this protein was identical to the GRA-2 antigen, which is expressed in both the tachyzoite and bradyzoite stages of the parasite. Recurrent ocular toxoplasmosis is thought to be due to reactivation of tissue cysts within the retina. Klaren \textit{et al.} suggest that it is possible that the intraocular immune response is partly directed against antigens from these cysts. \[^{80}\]. Assuming that the initial systemic infection leads to priming of the immune system against the tachyzoite stage of the antigen it is possible that local antibody response seen in patients with chronic recurring ocular toxoplasmosis is in response to antigens common to both the bradyzoite and tachyzoite stages of the parasite. Thus it could be hypothesised that the ocular immune response seen in patients with chronic recurrent ocular toxoplasmosis is in response to antigens leaking from cysts \[^{80}\]. If this, is the case it would not require the presence of tachyzoites in the anterior chamber to elicit such a response, and hence by implication there would not be any DNA therein.

\textit{In Conclusion}

The B1 PCR protocol appears to be not only highly specific in the amplification of \textit{T. gondii} DNA but also in our hands, the most sensitive protocol in the detection of \textit{T. gondii} and has been successful in the identification of \textit{T. gondii} DNA in both vitreous and retinal sections.
PCR on ocular samples remains a useful diagnostic aid in those cases of inflammatory ocular disease where the diagnosis is in doubt, especially where the aetiological agent is suspected to be an herpetic virus. However where ocular toxoplasmosis is suspected, we conclude that aqueous is not the appropriate ocular fluid from which to use PCR to amplify *T. gondii* DNA and that an alternative approach, perhaps using PCR analysis of vitreous samples in such cases might be more successful.
Table 9. B1 gene Primer sequences

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<th>Sequence</th>
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<tbody>
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<td>Outer primer (sense strand)</td>
<td>5'-GGAACTGCATCCGTTTCATGAG-3'</td>
<td>694-714</td>
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<td>5'-TCTTTAAAGCGGTTGCTGGTC-3'</td>
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<tr>
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<td>5'-TGCATAGGGTTGCGACTGAGTCTG-3'</td>
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Table 10. P30 gene primer sequences

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<td>Outer primer (nonsense strand)</td>
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<td>Inner Primer (sense strand)</td>
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<td>Inner Primer(nonsense strand)</td>
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Table 11. Ribosomal gene primer sequences

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<td>Non sense strand primer</td>
<td>5'-TAGGCATTCCGGTTAAAGATT-3'</td>
<td>253-231</td>
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Figure 2-1 Amplification of 193bp B1 PCR product from purified genomic DNA detects 50 fg *T. gondii* DNA

Varying quantities of *T. gondii* genomic DNA were subject to PCR amplification using B1-oligonucleotide primers 5'-GGAACCTGCATCCGTTCATGAG-3' and 5'-TCTTTAAAGCGTTCGTGGTC-3'. Lane 1, Molecular size marker (Gibco BRL, Paisley, Scotland), Lane 2, 1 ng *T. gondii* genomic DNA, Lane 3, 100 pg *T. gondii* genomic DNA, Lane 4, 10 pg *T. gondii* genomic DNA, Lane 5, 1 pg *T. gondii* genomic DNA Lane 6, 100 fg *T. gondii* genomic DNA, Lane 7, 50 fg *T. gondii* genomic DNA, Lane 8, No *T. gondii* genomic DNA (negative control). PCR products (10 μl) were resolved on 2% agarose/TBE gels and visualised following ethidium bromide staining under UV illumination.

Figure 2-2 Nested amplification of 96bp product from 193 bp product generated from purified genomic DNA detects 50 fg *T. gondii* DNA

Amplification of 96 bp PCR product from 193 bp product originally generated from *T. gondii* genomic DNA was achieved using nested primers 5'-TGCATAGGTTGCAGTCACTG-3' and 5'-GGCGACCAATCTGGAATACACC-3'. Lane 1, Molecular size marker (Gibco BRL, Paisley, Scotland). PCR products used for nested amplification were originally amplified from: Lane 2, 1 ng *T. gondii* genomic DNA, Lane 3, 100 pg *T. gondii* genomic DNA, Lane 4, 10 pg *T. gondii* genomic DNA Lane 5, 1 pg *T. gondii* genomic DNA, Lane 6, 100 fg *T. gondii* genomic DNA, Lane 7, 50 fg *T. gondii* genomic DNA, Lane 8, First round negative control after nested reaction, Lane 9, Nested negative control. PCR products (10 μl) were resolved on 2% agarose/TBE gels and visualised following ethidium bromide staining under UV illumination.
Figure 2-3: Amplification of 193bp B1 PCR product from purified genomic DNA detects 1pg *T. gondii* DNA in the presence of 20% normal human aqueous.

Varying quantities of *T. gondii* genomic DNA were subject to PCR amplification using specific B1-oligonucleotide primers 5'-GGAAC GATCCGTTTCA TGAAG-3' and 5'-TCTTT AAGCCTCGTGTC-3'. Lane 1, Molecular size marker (Gibco BRL, Paisley, Scotland), Lane 2, 1ng *T. gondii* genomic DNA, Lane 3, 100pg *T. gondii* genomic DNA, Lane 4, 10pg *T. gondii* genomic DNA, Lane 5, 1pg *T. gondii* genomic DNA, Lane 6, 100fg *T. gondii* genomic DNA, Lane 7, 50fg *T. gondii* genomic DNA, Lane 8, No *T. gondii* genomic DNA (negative control). PCR products (10μl) were resolved on 2% agarose/TBE gels and visualised following ethidium bromide staining under UV illumination.

Figure 2-4: Nested amplification of 96bp product from 193bp B1 PCR product generated from purified genomic DNA detects 50 fg *T. gondii* DNA in the presence of 20% normal human aqueous.

Amplification of 96 bp PCR product from 193 bp product originally generated from varying quantities of *T. gondii* genomic DNA was achieved using nested primers 5'-GCGGATGTTGCAGTC ACTG-3' and 5'-GCACCAAATCTGGGAATA CACC-3'. Lane 1, Molecular size marker (Gibco BRL, Paisley, Scotland). PCR products used for nested amplification were originally amplified from: Lane 2, 100pg *T. gondii* genomic DNA, Lane 3, 10pg *T. gondii* genomic DNA, Lane 4, 1pg *T. gondii* genomic DNA, Lane 5, 100fg *T. gondii* genomic DNA, Lane 6, 50fg *T. gondii* genomic DNA, Lane 7, 10fg *T. gondii* genomic DNA, Lane 8, First round negative control after nested reaction, Lane 9, Nested negative control. PCR products (10μl) were resolved on 2% agarose/TBE gels and visualised following ethidium bromide staining under UV illumination.
Figure 2-5 Amplification of 914bp P30 PCR product from purified genomic DNA detects 1pg *T. gondii* DNA

Varying quantities of *T. gondii* genomic DNA were subject to PCR amplification using specific P30-oligonucleotide primers 5'-TGCCGCGCCACACTGATG-3' and 5'-CGCGACACAAGCTGCGATAG-3'. Lane 1, Molecular size marker (Promega UK Ltd., Southampton, UK), Lane 2, Ing *T. gondii* genomic DNA, Lane 3, 100pg *T. gondii* genomic DNA, Lane 4, 10pg *T. gondii* genomic DNA, Lane 5, 1pg *T. gondii* genomic DNA, Lane 6, 100fg *T. gondii* genomic DNA, Lane 7, 50 fg *T. gondii* genomic DNA, Lane 8, No *T. gondii* genomic DNA (negative control). PCR products (10μl) were resolved on 1% agarose/TBE gels and visualised following ethidium bromide staining under UV illumination.

Figure 2-6. Nested amplification of 522bp product from 914bp P30 PCR product generated from purified genomic DNA detects 50fg *T. gondii* DNA

Amplification of 522 bp PCR product from 914 bp product originally generated from varying quantities of *T. gondii* genomic DNA was achieved using nested primers 5'-CGACAGCCGGTCATTCTC-3' and 5'-GCAACCAGTCAGCGTGCTCC -3'. Lane 1, Molecular size marker (Promega UK Ltd, Southampton, UK). PCR products used for nested amplification were originally amplified from: Lane 2, 1ng *T. gondii* genomic DNA, Lane 3, 100pg *T. gondii* genomic DNA, Lane 4, 10pg *T. gondii* genomic DNA, Lane 5, 1pg *T. gondii* genomic DNA, Lane 6, 100fg *T. gondii* genomic DNA, Lane 7, 50fg *T. gondii* genomic DNA, Lane 8, First round negative control after nested reaction, Lane 9, Nested negative control. PCR products (10μl) were resolved on 1% agarose/TBE gels and visualised following ethidium bromide staining under UV illumination.
Figure 2-7: Amplification of 914bp P30 PCR product from purified genomic DNA detects 1ng *T. gondii* DNA in the presence of 20% normal human aqueous.

Varying quantities of *T. gondii* genomic DNA were subject to PCR amplification using specific P30-oligonucleotide primers 5'-TTGCCGCGCCACACTGATG-3' and 5'-CGCGACACAAGCTGCGATAG-3'. Lane 1, Molecular size marker (Promega UK Ltd., Southampton, UK), Lane 2, 1ng *T. gondii* genomic DNA, Lane 3, 100pg *T. gondii* genomic DNA, Lane 4, 10pg *T. gondii* genomic DNA, Lane 5, 1pg *T. gondii* genomic DNA, Lane 6, 100fg *T. gondii* genomic DNA, Lane 7, 50fg *T. gondii* genomic DNA, Lane 8, No *T. gondii* genomic DNA (negative control). PCR products (10μl) were resolved on 1% agarose/TBE gels and visualised following ethidium bromide staining under UV illumination.

Figure 2-8 Nested amplification of 522bp product from 914bp P30 PCR product generated from purified genomic DNA only detects 1pg *T. gondii* DNA in the presence of 20% normal human aqueous.

Amplification of 522 bp PCR product from 914 bp template originally generated from varying quantities of *T. gondii* genomic DNA was achieved using nested primers 5'-CGACAGCCGCCTATTCTC-3' and 5'-GCAAAGTCAAGCGTCC-3'. Lane 1, Molecular size marker (Promega UK Ltd., Southampton, UK). PCR products used for nested amplification were originally amplified from: Lane 2, 1ng *T. gondii* genomic DNA, Lane 3, 100pg *T. gondii* genomic DNA, Lane 4, 10pg *T. gondii* genomic DNA, Lane 5, 1pg *T. gondii* genomic DNA, Lane 6, 100fg *T. gondii* genomic DNA, Lane 7, 50fg *T. gondii* genomic DNA, Lane 8, First round negative control after nested reaction, Lane 9, Nested negative control. PCR products (10μl) were resolved on 1% agarose/TBE gels and visualised following ethidium bromide staining under UV illumination.
Figure 2-9 Amplification of an 88bp PCR product from the 18S rDNA gene from purified genomic DNA detects 1pg T. gondii DNA

Varying quantities of T. gondii genomic DNA were subject to PCR amplification using specific 18S rDNA oligonucleotide primers 5'-CCTTGGCCGATAGGTCTAGG-3' and 5'-TAGGCATTCGGGTTAAAGATTA-3'. Lane 1, Molecular size marker (Gibco BRL, Paisley, Scotland), Lane 2, 1ng T. gondii genomic DNA, Lane 3, 100pg T. gondii genomic DNA, Lane 4, 10pg T. gondii genomic DNA, Lane 5, 1pg T. gondii genomic DNA, Lane 6, 100fg T. gondii genomic DNA, Lane 7, 50fg T. gondii genomic DNA, Lane 8, No T. gondii genomic DNA (negative control). PCR products (20µl) were resolved on 4% TBE / metaphor agarose gels (Flowgen Instruments, Lichfield, UK) and visualised following ethidium bromide staining under UV illumination.

Figure 2-10 Amplification of an 88bp PCR product from the 18S ribosomal DNA gene from purified genomic DNA only detects 1pg T. gondii DNA in the presence of 20% normal human aqueous.

Varying quantities of T. gondii genomic DNA were subject to PCR amplification using specific 18S rDNA oligonucleotide primers 5'-CCTTGGCCGATAGGTCTAGG-3' and 5'-TAGGCATTCGGGTTAAAGATTA-3'. Lane 1, Molecular size marker (Gibco BRL, Paisley, Scotland), Lane 2, 1ng T. gondii genomic DNA, Lane 3, 100pg T. gondii genomic DNA, Lane 4, 10pg T. gondii genomic DNA, Lane 5, 1pg T. gondii genomic DNA, Lane 6, 100fg T. gondii genomic DNA, Lane 7, 50fg T. gondii genomic DNA, Lane 8, No T. gondii genomic DNA (negative control). PCR products (20µl) were resolved on 4% TBE / metaphor agarose gels (Flowgen Instruments, Lichfield, UK) and visualised following ethidium bromide staining under UV illumination.
Figure 2-11: Amplification of 193bp B1 PCR product from 1ng purified *T. gondii* genomic DNA in 20% normal human aqueous in the presence of human lymphocyte DNA.

1ng *T. gondii* genomic DNA in a background of increasing amounts of human genomic DNA were subject to PCR amplification using specific B1-oligonucleotide primers 5'-GGAACTGCATCCGTTCATGAG-3' and 5'-TCTTTAAAGCGTTCGTGGTC-3'. Lane 1, Molecular size marker (Gibco BRL, Paisley, Scotland), Lane 2, 1ng *T. gondii* genomic DNA, plus 10ng human DNA, Lane 3, 1ng *T. gondii* genomic DNA plus 50ng human DNA, Lane 4, 1ng *T. gondii* genomic DNA, plus 100 ng human DNA, Lane 5, 1ng *T. gondii* genomic DNA, plus 150 ng human DNA, Lane 6, No DNA (negative control), Lane 7 150ng human DNA only. Lane 8, 1ng *T. gondii* genomic DNA only (positive control). PCR products (10μl) were resolved on 2% agarose/TBE gels and visualized following ethidium bromide staining under UV illumination.

Figure 2-12 Nested amplification of 96bp product from 193bp B1 PCR product generated from 1ng purified genomic *T. gondii* DNA in 20% normal human aqueous in the presence of human lymphocyte DNA.

Amplification of 96 bp PCR product from 193 bp product originally generated from 1ng *T. gondii* genomic DNA was achieved using nested primers 5'-TGCATAGGTTGCAGTCACTG-3' and 5'-GGCGACCAAATCTGGAATACACC-3'. Lane 1, Molecular size marker (Gibco BRL, Paisley, Scotland). PCR products used for nested amplification were originally amplified from: Lane 2, 1ng *T. gondii* genomic DNA, plus 10 ng human DNA, Lane 3, 1ng *T. gondii* genomic DNA, plus 50 ng human DNA, Lane 4, 1ng *T. gondii* genomic DNA, plus 100 ng human DNA, Lane 5, 1ng *T. gondii* genomic DNA, plus 150ng human DNA, Lane 6, 1ng *T. gondii* genomic DNA only (first round positive control), Lane 7, 150ng human DNA only (nested negative control) Lane 8 1ng *T. gondii* genomic DNA, (nested positive control),Lane 9, Nested negative control (water). PCR products (10μl) were resolved on 2% agarose/TBE gels and visualised following ethidium bromide staining under UV illumination.
Figure 2-13: First Round Amplification of 193bp B1 PCR product from 1ng T. gondii genomic DNA in inflamed aqueous.

1ng T.gondii genomic DNA spiked into different quantities of inflamed aqueous were subject to PCR amplification using specific B1-oligonucleotide primers 5'-GGAACTGCATCCGTTCATGAG-3' and 5'-TCTTTAAAGCGTTCGTGGTC-3'. Lane 1, Molecular size marker (Gibco BRL, Paisley, Scotland), Lane 2, 1ng T. gondii genomic DNA in 1µl aqueous, Lane 3, 1ng T. gondii genomic DNA in 5µl aqueous, Lane 4, 1ng T. gondii genomic DNA in 10µl aqueous, Lane 5, 1ng T. gondii genomic DNA in 15µl aqueous, Lane 6, 1ng T. gondii genomic DNA in water (positive control), Lane 7, No T. gondii genomic DNA (negative control). PCR products (10µl) were resolved on 2% agarose/TBE gels and visualised following ethidium bromide staining under UV illumination.

Figure 2-14 Nested amplification of 96bp B1 PCR product generated from 1ng purified genomic T. gondii DNA in inflamed human aqueous.

Amplification of 96 bp PCR product from 193 bp product originally generated from 1 ng T. gondii genomic DNA was achieved using nested primers 5'-TGCAATGTGGCTGCTGTGCTG-3' and 5'-GGCGAACCATTGCAATGGCAATACACC-3'. Lane 1, Molecular size marker (Gibco BRL, Paisley, Scotland). PCR products used for nested amplification were originally amplified from: Lane 2, 1ng T. gondii genomic DNA in 1µl aqueous, Lane 3, 1ng T. gondii genomic DNA in 5µl aqueous, Lane 4, 1ng T. gondii genomic DNA in 10µl aqueous, Lane 5, 1ng T. gondii genomic DNA in 15µl aqueous, Lane 6, 1ng T. gondii genomic DNA in water (outer positive control), Lane 7, First round negative control after nested PCR. Additionally, Lane 8, 1ng T. gondii genomic DNA second round amplification only (second round positive control), Lane 9, Nested negative control. PCR products (10µl) were resolved on 2% agarose/TBE gels and visualised following ethidium bromide staining under UV illumination.
Figure 2-15 Amplification of 193bp B1 PCR product from purified genomic DNA detects 1pg *T. gondii* DNA in the presence of 20% inflamed human aqueous.

Varying quantities of *T. gondii* genomic DNA were subject to PCR amplification using specific B1-oligonucleotide primers 5'-GGAACTGCATCGTCATGAG-3' and 5'-TCTTTAAACGCTCTGCTGGTC-3'. Lane 1, Molecular size marker (Gibco BRL, Paisley, Scotland), Lane 2, 1ng *T. gondii* genomic DNA, Lane 3, 100pg *T. gondii* genomic DNA, Lane 4, 10pg *T. gondii* genomic DNA, Lane 5, 1pg *T. gondii* genomic DNA, Lane 6, 100fg *T. gondii* genomic DNA, Lane 7, 50fg *T. gondii* genomic DNA, Lane 8, No *T. gondii* genomic DNA (negative control). PCR products (10μl) were resolved on 2% agarose/TBE gels and visualized following ethidium bromide staining under UV illumination.

Figure 2-16 Nested amplification of 96bp product from 193bp B1 PCR product generated from purified genomic DNA detects 50 fg *T. gondii* DNA in the presence of 20% inflamed human aqueous.

Amplification of 96 bp PCR product from 193 bp product originally generated from varying quantities of *T. gondii* genomic DNA was achieved using nested primers 5'-TGATAGTTGGTGTCACT-3' and 5'-GGCGACCAATCTGCGAATACC-3'. Lane 1, Molecular size marker (Gibco BRL, Paisley, Scotland). PCR products used for nested amplification were originally amplified from: Lane 2, 1ng *T. gondii* genomic DNA, Lane 3, 100pg *T. gondii* genomic DNA, Lane 4, 10pg *T. gondii* genomic DNA, Lane 5, 1pg *T. gondii* genomic DNA, Lane 6, 100fg *T. gondii* genomic DNA, Lane 7, 50fg *T. gondii* genomic DNA, Lane 8, First round negative control after nested reaction, Lane 9, Nested negative control. PCR products (10μl) were resolved on 2% agarose/TBE gels and visualized following ethidium bromide staining under UV illumination.
Figure 2-17: Nested PCR using Primers directed against the B1 gene.

Comparison of direct PCR to PCR with DNA extraction proceeded by heating at 95°C for 20 minutes.

A) Direct PCR: Amplification of 193bp B1 PCR product detects 4.6x10^3 tachyzoites
Known quantities of *T. gondii* tachyzoites in aqueous were subject to PCR amplification. Lane 1, 4.6x10^3 *T. gondii* tachyzoites, Lane 2, 4.6x10^4 *T. gondii* tachyzoites, Lane 3, 4.6x10^5 *T. gondii* tachyzoites

B) Direct PCR: Nested amplification of 96bp product from 193bp B1 PCR product detects 46 tachyzoites
PCR products used for nested amplification were originally amplified from: Lane 1, 4.6x10^3 *T. gondii* tachyzoites, Lane 2, 4.6x10^4 *T. gondii* tachyzoites, Lane 3, 4.6x10^5 *T. gondii* tachyzoites, Lane 4, 4.6x10^6 *T. gondii* tachyzoites, Lane 5, 46 *T. gondii* tachyzoites.

C) Heating at 95°C for 20 minutes prior to PCR. Amplification of 193bp B1 PCR product detects 46 tachyzoites
Known quantities of *T. gondii* tachyzoites in aqueous were subject to PCR amplification. Lane 1, 4.6x10^3 *T. gondii* tachyzoites, Lane 2, 4.6x10^4 *T. gondii* tachyzoites, Lane 3, 4.6x10^5 *T. gondii* tachyzoites, Lane 4, 4.6x10^6 *T. gondii* tachyzoites, Lane 5, 46 *T. gondii* tachyzoites.

D) Heating at 95°C for 20 minutes prior to PCR: Nested amplification of 96bp product from 193bp B1 PCR product detects less than a single tachyzoite
PCR products used for nested amplification were originally amplified from: Lane 1, 4.6x10^3 *T. gondii* tachyzoites, Lane 2, 4.6x10^4 *T. gondii* tachyzoites, Lane 3, 4.6x10^5 *T. gondii* tachyzoites, Lane 4, 4.6x10^6 *T. gondii* tachyzoites, Lane 5, 46 *T. gondii* tachyzoites, Lane 6, 4.6 *T. gondii* tachyzoites, Lane 7, 4.6x10^3 *T. gondii* tachyzoites.
Figure 2-18: First Round Amplification of 193bp B1 PCR product from vitreous obtained from a patient with T. gondii retinochoroiditis.

5μl quantities of a vitreous sample from a patient with T.gondii retinochoroiditis were subject to PCR amplification using specific B1-oligonucleotide primers 5'-GGAACTGCATCCGTTCATGAG-3' and 5'-TCTTTAAAGCGTTCGTGGTC-3'. Lane 1, Molecular size marker (Gibco BRL, Paisley, Scotland), Lane 2, vitreous sample spiked with Ing T. gondii genomic DNA, Lane 3, vitreous sample spiked with lpg T. gondii genomic DNA, Lane 4, vitreous sample spiked with 100fg T. gondii genomic DNA, Lane 5, vitreous sample only (no added T.gondii DNA). PCR products (10μl) were resolved on 2% agarose/TBE gels and visualised following ethidium bromide staining under UV illumination.

Figure 2-19: Nested amplification of 96bp B1 PCR product generated from vitreous obtained from a patient with T. gondii retinochoroiditis.

Amplification of 96 bp PCR product from vitreous sample from a patient with T. gondii retinitis was achieved using nested primers 5'-TGCATAGGTTGCAGTCACTG-3' and 5'-GGCGACCAATCTGCGAATACACC-3'. PCR products used for nested amplification were originally amplified from: Lane 1, Vitreous sample spiked with lng T. gondii genomic DNA. Lane 2, Vitreous sample spiked with lpg T. gondii genomic DNA, Lane 3, Vitreous sample spiked with 100fg T. gondii genomic DNA Lane 4, Vitreous sample only (no added T.gondii DNA) PCR products (10μl) were resolved on 2% agarose/TBE gels and visualised following ethidium bromide staining under UV illumination.
Figure 2-20: First Round Amplification of 193bp B1 PCR product from deparaffinised retinal sections obtained from a patient with *T. gondii* retinochoroiditis.

10μl quantities of supernatant from deparaffinised retinal sections from a patient with *T. gondii* retinochoroiditis that had undergone protein extraction were subject to PCR amplification using specific B1-oligonucleotide primers 5'-GGAACTGCATCCGTTCATGAG-3' and 5'-TCTTTAAAGCGTTCGTGGTC-3'. Lanes 1, 2, and 3, supernatant from first retinal section that had not been further diluted (Lane 1) or had been diluted 1 in 2 (Lane 2) or 1 in 10 (Lane 3). Lanes 4, 5, and 6, supernatant from second retinal section that had not been further diluted (Lane 4) or had been diluted 1 in 2 (Lane 5) or 1 in 10 (Lane 6). Lanes 7, 8, and 9, supernatant from third retinal section that had not been further diluted (Lane 7) or had been diluted 1 in 2 (Lane 8) or 1 in 10 (Lane 9). Lanes 10, 11, and 12, supernatant from fourth retinal section that had not been further diluted (Lane 10) or had been diluted 1 in 2 (Lane 11) or 1 in 10 (Lane 12). PCR products (10μl) were resolved on 2% agarose/TBE gels and visualised following ethidium bromide staining under UV illumination.

Figure 2-21: Control samples used during First Round Amplification of 193bp B1 PCR product from deparaffinised retinal sections obtained from a patient with *T. gondii* retinochoroiditis.

Lane 1-3, *T. gondii* positive extraction controls; 10ng genomic *T. gondii* DNA spiked into undiluted supernatant (Lane 1) and supernatant that had been diluted 1 in 2 (Lane 2) and 1 in 10 (Lane 3). Lane 4 and 5; blank. Lanes 6, 7, and 8 negative extraction controls (supernatant from extraction with no added *T. gondii* DNA); undiluted, (Lane 6) and diluted 1 in 2 (Lane 7) and diluted 1 in 10 (Lane 8). Lane 9; blank. Lane 10; 10ng *T. gondii* DNA spiked into sterile water; PCR positive control. Lane 11; blank. Lane 12; sterile water, no added DNA; PCR negative control. PCR products (10μl) were resolved on 2% agarose/TBE gels and visualised following ethidium bromide staining under UV illumination.
Figure 2-22: Nested amplification of 96bp B1 PCR product generated from deparaffinised retinal sections obtained from a patient with *T. gondii* retinochoroiditis.

Amplification of 96 bp PCR product from deparaffinised retinal sections from a patient with *T. gondii* retinitis was achieved using nested primers 5'-TGCATAGGTTGAGTCGCTG-3' and 5'-GGCGACCAATCTGGAATACACC-3'. PCR products used for nested amplification were originally amplified from: Lane 1, supernatant from first retinal section that had not been further diluted or had been diluted 1 in 2 (lane2) or 1 in 10 (Lane 3). Lane 4, supernatant from second retinal section that had not been further diluted or had been diluted 1 in 2 (lane5) or 1 in 10 (Lane 6). Lane 7, supernatant from third retinal section that had not been further diluted or had been diluted 1 in 2 (lane8) or 1 in 10 (Lane 9). Lane 10, supernatant from third retinal section that had not been further diluted or had been diluted 1 in 2 (lane11) or 1 in 10 (Lane 12). PCR products (10µl) were resolved on 2% agarose/TBE gels and visualised following ethidium bromide staining under UV illumination.
Chapter 3: Changes in the ocular manifestations in AIDS patients since the introduction of highly active anti-retroviral therapy (HAART)

Introduction

Does combination anti-retroviral therapy change survival of AIDS patients with CMV retinitis?

Introduction

The introduction of highly active antiretroviral therapy, (HAART), and more recently combinations including HIV proteinase inhibitors (PIs), has been associated with great improvements in the survival of people with HIV disease. However it remains unclear whether the immune deficit is wholly reversible [513]. Hence patients with manifestations of severe chronic immunodeficiency such as cytomegalovirus (CMV) retinitis might be expected to derive least benefit from this form of therapy. A longitudinal study of patients with CMVR diagnosed between October 1992 and May 1996 and followed up to May 1997 was undertaken to to assess the effect of HAART including PIs on the survival of patients with cytomegalovirus retinitis (CMVR). The outcome measure used was time to death from first diagnosis of CMVR. Data were censored on 31st May 1997.
Methods

All HIV-1 seropositive patients attending our centre who were at risk of CMVR were assessed by an experienced ophthalmologist using indirect ophthalmoscopy. This included all patients with symptoms or signs suggestive of CMVR, patients with extra-ocular CMV disease and also asymptomatic patients with a CD4+ lymphocyte count <50 cells/μl who were clinically examined for CMVR in the same way at three monthly intervals. CMVR was diagnosed by an experienced ophthalmologist by the presence of the characteristic progressive, white, fluffy or granular retinal infiltrates, with or without associated haemorrhage [514, 515]. Cases of CMVR were identified primarily from a computer database into which all AIDS diagnoses made in our centre have been entered prospectively. Completeness of case finding was verified by cross-referencing pharmacy records for prescriptions of foscarnet, cidofovir and ganciclovir. The diagnosis of CMVR was then confirmed from entries in the case records.

We reviewed the case records of all such patients diagnosed with CMVR between 1st October 1992 and 31st May 1996. Data were abstracted on date of birth, sex, HIV risk behavior, date first tested HIV-1 seropositive, date of first AIDS defining diagnosis [516] date of first CMVR diagnosis and date of death. Date of death was recovered from the computer database: a date of death was only entered into this database if death occurred as an inpatient at our centre, or if the date had been confirmed by the patient’s general practitioner or local health authority. Where no such date was available or where there had been no contact with the patient for more than two months prior to death the patient was considered lost to follow up. Drug
histories were obtained relating to anti-CMV medication, *Pneumocystis carinii* pneumonia (PCP) prophylaxis and antiretroviral therapy. All drug histories related to treatments received following first diagnosis of CMVR unless otherwise stated. Data were censored at 31st May 1997.

Distribution of continuous variables was analysed by Student’s *t*-test. Rank correlation of non-parametric data was studied using Spearman’s ρ-test. χ² test was used for categorical analysis where appropriate. The Kaplan-Meier estimate of cumulative survival distribution was used to calculate median or (where the number of events did not reach 50% for any subgroup) mean survival times; the effect of single variables on survival was analysed by the log rank test. Multivariate analysis was performed by the Cox regression method with date of first CMV retinitis diagnosis as a time dependent co-variate. Changes in survival over time were analysed cross-sectionally as the time from CMV retinitis diagnosis to death in patients alive with CMV retinitis at serial date intervals over the study period. Statistical significance was achieved if p<0.05 and all significance tests were two tailed. Statistical analysis was performed using SPSS for Windows software (release 7.0).
Results

A diagnosis of CMVR was made in 155 patients; 8 patients were lost to follow up and were excluded from further analysis. Demographic and immunological data for 147 patients followed to 31st May 1997 (Table 12). Date of first CMVR diagnosis ranged from 21st October 1992 to 1st May 1996.

Table 12: Demographic, treatment and immunological variables.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Entire cohort</th>
<th>No PI</th>
<th>PI Treated</th>
<th>p=</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>147</td>
<td>123</td>
<td>24</td>
<td>-</td>
</tr>
<tr>
<td>Males (%)</td>
<td>95.2</td>
<td>95.8</td>
<td>95.1</td>
<td>N.S.†</td>
</tr>
<tr>
<td>HIV risk group* (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homosexual males:</td>
<td>89.2</td>
<td>89.7</td>
<td>86.4</td>
<td>N.S.†</td>
</tr>
<tr>
<td>Intravenous drug users:</td>
<td>6.5</td>
<td>6.0</td>
<td>9.1</td>
<td></td>
</tr>
<tr>
<td>Sub-Saharan Africans:</td>
<td>3.6</td>
<td>3.4</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>Mean age ± 1 s.d. (years)§</td>
<td>38.4±7.9</td>
<td>38.4±8.2</td>
<td>38.2±6.4</td>
<td>N.S.‡</td>
</tr>
<tr>
<td>Mean CD4 count (range) (cells/µl)§</td>
<td>11.7 (0-77)</td>
<td>12.0 (0-77)</td>
<td>10.5 (2-34)</td>
<td>N.S.‡</td>
</tr>
<tr>
<td>Mean days on foscarnet (range)</td>
<td>50.0 (0-336)</td>
<td>48.5 (0-322)</td>
<td>59.0 (0-336)</td>
<td>N.S.‡</td>
</tr>
<tr>
<td>Received ART before CMVR (%)</td>
<td>72.7</td>
<td>71.5</td>
<td>79.2</td>
<td>N.S.†</td>
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<tr>
<td>Alive 31st May 1997 (%)</td>
<td>12.2</td>
<td>0.0</td>
<td>75.0</td>
<td>-</td>
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</table>

*Individuals may have belonged to more than 1 group

† χ² test

‡ Student’s t-test

§ at CMVR diagnosis
The median time from first CMVR diagnosis to death for the entire cohort was 262 days (95% CI 195-329 days), a 1 year survival of 35.4%. The median survival of all patients alive with CMVR before PIs were first introduced in December 1995 was 256 days (n=134; 95%CI 197-315 days). In the group including all patients alive with CMVR from 1st December 1995 onwards median survival was 555 days (n=49; 95%CI 351-759 days) irrespective of antiretroviral therapy received. Indeed by 31st May 1996 median survival for the entire group of patients alive with CMVR at this time had risen to 720 days (95%CI 551-889 days).

CMV retinitis patients were prescribed PIs from December 1995 (Figure 3-1). Indinavir was used in 58% of these cases at some time, saquinavir in 54%, nelfinavir in 4% and dual saquinavir and ritonavir in 25%; all were prescribed in combination with nucleoside reverse transcriptase inhibitors (NRTIs). In one case initiation of PI based therapy preceded CMV retinitis diagnosis by 38 days. In the remaining 23 patients who received a PI median time from CMV retinitis to initiation of PI was 255 days (range 44-829 days). Of the 123 patients who never received a PI, 22 took NRTI monotherapy only and 12 took dual NRTI therapy after CMV retinitis diagnosis. Twenty-four patients were alive with CMV retinitis after December 1995 but never received a PI: 12 died before PIs became generally available in April 1996 and a further 8 within 2 months of this. For the remaining 4 patients there was no recorded reason why PI therapy was not initiated. All patients were prescribed PCP prophylaxis.
Patients diagnosed with CMV retinitis later in the study period spent significantly more time on HAART after CMV retinitis was diagnosed than earlier patients (Spearman’s ρ = 0.311, p<0.001). In bivariate analysis the mean survival of those who took no further HAART after CMV retinitis diagnosis was 224 days (95% CI 186-261 days; 1 year survival 16%) compared with 353 days in those who took NRTIs but no PI (95% CI 289-418 days; 1 year survival 50%). However among those who took a PI mean survival was 914 days (95% CI 768-1059 days; one-year survival 83%). This difference was highly significant (p<0.0001) (Figure 3-2).

Time to death from first CMV retinitis diagnosis was significantly longer among those for whom CMV retinitis was the first AIDS defining event than in those with previous AIDS diagnoses. However, when time to death from first AIDS diagnosis was examined, patients whose first AIDS diagnosis was CMV retinitis had a significantly shorter survival (Table 13). CD4+ lymphocyte count at CMV retinitis diagnosis was similar in those who ever received a PI or not (Table 12). In those receiving a PI the mean CD4+ lymphocyte count rose to $65 \times 10^6$/ml (n=18) at 1 year and continued to rise out to 28 months ($132 \times 10^6$/ml, n=5, Pearson correlation coefficient $r=0.92$, $p=0.001$) while for those never receiving a PI it continued to fall ($6 \times 10^6$/ml at 1 year, n=10, $r=-0.94$; $p=0.006$).

Following an induction course, maintenance foscarnet was given at a dose of 120 mg/kg (adjusted for estimated creatinine clearance) once daily for two weeks then 5 days per week thereafter. Having ever received foscarnet ($p=0.044$) and having remained on foscarnet for over 1 month ($p=0.0052$) were both associated with increased survival by univariate analysis. The following factors were not associated
with a change in survival: date of first AIDS diagnosis, CD4+ lymphocyte count at first CMV retinitis diagnosis, exposure to anti-retrovirals before CMV retinitis diagnosis, age, sex and HIV risk group.

Table 13: Comparing survival in patients whose first AIDS diagnosis was CMV retinitis with patients having other first AIDS diagnoses.

<table>
<thead>
<tr>
<th>First AIDS diagnosis</th>
<th>CMVR</th>
<th>Other first AIDS diagnoses</th>
<th>p=</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>20</td>
<td>127</td>
<td>-</td>
</tr>
<tr>
<td>Number receiving PI</td>
<td>7*</td>
<td>17</td>
<td>0.024†</td>
</tr>
<tr>
<td>Comparison of days to death from first AIDS diagnosis (95% CI)</td>
<td>477 (220-734)</td>
<td>842 (746-938)</td>
<td>0.015</td>
</tr>
<tr>
<td>Comparison of days to death from CMVR diagnosis (95% CI)</td>
<td>477 (220-734)</td>
<td>218 (140-296)</td>
<td>0.002</td>
</tr>
</tbody>
</table>

* None were receiving a PI at the time of first CMVR diagnosis.

† $\chi^2$ test
Multivariate analysis revealed that independent predictors of improved survival were:

1) having ever received a PI after CMV retinitis diagnosis (p<0.0001, relative risk of death (RRD)= 0.063, 95% CI= 0.027-0.149),

2) having ever taken a NRTI after CMV retinitis diagnosis (p= 0.003, RRD = 0.52, 95% CI= 0.34-0.80) and

3) Foscarnet therapy for over 1 month (p= 0.04, RRD = 0.68, 95% CI= 0.48-0.98).

When type of HAART prescribed to individuals for the most time after CMV retinitis diagnosis was accounted for (PI containing combinations, NRTIs only or none), only remaining on a PI for the majority of time (p<0.0001, RRD = 0.04, 95% CI= 0.01-0.16) and first AIDS diagnosis being CMV retinitis (p= 0.0008, RRD = 0.37, 95% CI= 0.21-0.66) were predictive of improved survival. NRTI and foscarnet therapy did not achieve significance.
Discussion

HAART usually consists of a combination of a PI with two NRTIs.

The effects of HAART on the natural history of cytomegalovirus retinitis have been the subject of keen interest and debate from the earliest introduction of the protease inhibitors. Increased times to disease relapse have been reported in patients taking HAART. We have found a pronounced increase in median survival for the entire group of patients alive with CMV retinitis in the latter part of the study period, beyond that previously reported. This increase coincided with the introduction of PIs. Selection of fitter patients for PI therapy is unlikely to fully account for this finding as the cohort included all patients with CMV retinitis irrespective of treatment received. Indeed from the date when entry to the cohort closed (31st May 1996) the majority of patients alive with CMV retinitis received a PI (Figure 3-1).

Apart from PI based therapy, a number of other changes in our population and the management of their disease may have occurred over the study period and influenced survival. Multivariate analysis of identifiable factors revealed that by far the strongest association with increased survival was exposure to PI containing antiretroviral regimens. It is noteworthy that the majority of PI experienced cases remained alive at the censoring date (Figure 3-2) and hence we may have actually underestimated the true magnitude of the effect of PIs. Alternatively it might be argued that the improvement in survival was an artefact caused by a reduction in other first AIDS diagnoses due to PI therapy and hence a relative increase in CMV retinitis as the AIDS defining event. (Table 13) confirms that such a shift would lead
to longer survival after CMV retinitis. However this explanation is unsatisfactory as none of those with CMV retinitis as a first AIDS diagnosis was on a PI at the time.

This fall in mortality is consistent with the decline in AIDS related deaths in adults in the United States [518]. Hogg et al. recently reported falling mortality among patients with CD4 counts <100x10⁹/ml which coincided with increased prescription of lamivudine and dual NRTI therapy [519]. The number of patients in our study remaining on dual NRTI combinations for a long period was, however, too small to discern an effect. No overall benefit from NRTI monotherapy has been found in patients with CMV retinitis [335, 520, 521] and the survival of our patients who never received a PI parallels that previously reported. In common with previous studies [522-524], foscarnet therapy was associated with improved survival. The strength of this association was relatively weak and was lost when the data were analysed in terms of type of HAART prescribed for the majority of the time. This may imply that foscarnet therapy was merely associated with the ability to remain on antiretroviral therapy.

Despite increasing evidence that advanced HIV disease is associated with immune dysfunction that may not be wholly reversible even with potent antiretroviral therapy [513], our data suggest that this form of treatment may be useful even in individuals with severe immunodeficiency. According to Gallant et al. CMV disease contributes to mortality due to the combination of advanced immunodeficiency with inability to tolerate antiretroviral therapy [525]. The HIV-proteinase inhibitors offer new opportunities to find combinations that are both tolerable and sufficiently potent to reduce the mortality of CMV retinitis.
Figure 3-1: Changes in antiretroviral use after CMVR diagnosis over time. The period following first use of PIs in December 1995 is shown in greater detail.

Nil = no ART; MT = NRTI monotherapy; DT = NRTI dual therapy; PI = combination therapy with PI.
Figure 3-2: Kaplan-Meier survival curves for patients who received PI based combinations, NRTIs only or no further ART following CMVR diagnosis.
Chapter 4: What are the ocular manifestations of AIDS in rural communities in Central Africa

Historical Review of AIDS in Africa

The first confirmed AIDS cases in sub-Saharan Africans were diagnosed in Europe in 1983 [347]. Epidemiological studies on AIDS were conducted in Kinshasa, Zaire [526] and in Kigali, Rwanda [527] in 1983.

Several lines of evidence suggest that AIDS was uncommon in Africa before the mid-1970’s. Firstly, clinicians from several African countries have reported that, starting in the mid- to late 1970’s, unusual cases of what was assumed to be tuberculosis began to appear in increasing numbers. Secondly, marked increases in cases of indicator diseases, such as cryptococcal meningitis, chronic diarrhoea, and generalised Kaposi’s sarcoma, were observed in several central African cities in the late 1970’s and early 1980’s. Thirdly, reviews of hospital records identified very few cases of AIDS-like cases in Africans hospitalised in Europe before 1980. Finally, retrospective serological studies on stored blood have also shown that HIV infections began to spread in the large cities in Central Africa in the late 1970’s or early to mid 1980’s [347].

Incidence and Prevalence of HIV infection in Africa

AIDS in Central and East Africa is characterised epidemiologically by an approximately equal male to female ratio [281, 526]. This is in contrast to the nearly 19:1 male to female ratio in the United States [347]. The age specific peak of AIDS
incidence and HIV seroprevalence is between 20 and 39 years of age (with women generally being younger than men) [347].

**AIDS classification system in developing countries**

An alternative clinical classification has been proposed for use in developing countries, where CD4+ counts are rarely available [528]. This system categorises patients in four clinical groups based on clinical features of prognostic value (Table 14)

**Table 14 Clinical classification system for HIV infection in developing countries**

<table>
<thead>
<tr>
<th>Group 1</th>
<th>Asymptomatic or generalised lymphadenopathy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal Activity</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group 2</th>
<th>Early stage disease; a weight loss of less than 10% body weight, minor mucocutaneous manifestations, varicella zoster within 5 years, recurrent upper respiratory tract infections</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Symptomatic but normal activity</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group 3</th>
<th>Intermediate stage disease: weight loss of more than 10% body weight, unexplained chronic diarrhoea for more than 1 month, unexplained chronic fever for more than 1 month, oral candidiasis, oral hairy leucoplakia, pulmonary tuberculosis within 1 year, severe bacterial infections</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bed-ridden for less than 50% day during previous months</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group 4</th>
<th>AIDS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bed-ridden for more than 50% day during previous months</td>
</tr>
</tbody>
</table>

**Ocular Disease**

*HIV retinopathy*

There seems to be no difference in the incidence of HIV related cotton wool spots in African and American patients [281]. In addition, perivasculitis of the retinal vessels
with the AIDS-related complex seen in African children is also considered to be part of noninfectious AIDS retinopathy [390].

**Herpes Zoster**

Herpes zoster is a marker for HIV infection in Africa [529, 530] with a high positive predictive value. In the HIV positive population the disease tends to be more severe both in terms of corneal involvement and post-herpetic pain [292, 299]. The natural history of the disease tends to be more severe in the African population than in the Western population [292].

**Viral Diseases of the Eyelids**

Both molluscum contagiosum [531] and verrucae of the eyelids are common cutaneous manifestations of HIV infection in the tropics [281]. Mollusca contagiosum are caused by the pox virus [531] and are very common in young children in Africa [281].

**Choroidal tuberculosis**

Since the advent of HIV/AIDS there has been a large rise in the prevalence of tuberculosis (TB) [281], and in sub-Saharan Africa, TB is the most common infection in patients who die from AIDS [347, 532]. In Rwanda, examination of 32 HIV positive patients with TB revealed 5 (15.6%) with ocular lesions due to TB, including disseminated choroiditis, phlyctenulosis, and solitary granulomas [281]. In Malawi, however, examination of 68 HIV positive TB patients revealed only one with ocular disease which was probably TB related [533].

**Cytomegalovirus retinitis**

The frequency of CMV retinitis in African AIDS patients seems to be much lower than in Europe and the United States [533-535]. The shorter life expectancy of AIDS
patients in developing countries probably reduces the period of profound
immunodeficiency during which patients are likely to develop CMV retinitis [281],

Cryptococcal Infection

Cryptococcal meningitis is more common in African patients with AIDS than in
patients in Western Societies [536], probably because of the prevalence of C. neoformans in the domestic environment [537]. In HIV positive patients with
cryptococcal infection in Rwanda, papilloedema was observed in 32.5%, visual loss
in 9%, sixth cranial nerve palsy in 9% and optic atrophy in 2.5%. Actual invasion of
the intraocular structures with Cryptococcus neoformans was an uncommon
complication in these patients [288].

Neoplasia

Lymphoma

Although orbital lymphoma occurs in Africa, it is uncommon [281].

Squamous cell carcinoma of the conjunctiva

Following case reports of conjunctival squamous-cell carcinoma in HIV positive
men in the USA [538, 539], a striking increase in the number of patients with
conjunctival neoplasms were reported from Rwanda, Malawi, Uganda [281], and
Tanzania [540]. HIV infection is a risk factor for the development of squamous cell
dysplasia and neoplasia of the conjunctiva [541]. Data from Rwanda [541], Malawi
[542] and Uganda [543] indicate that approximately 77-80% of these patients are HIV
positive.

Cutaneous hypersensitivity reactions

Stevens-Johnson syndrome is part of a spectrum of skin and generalised disease due
to a hypersensitivity reaction to a variety of drugs. Sulpha drugs have been strongly
implicated. A study in Kenya demonstrated that TB patients infected with HIV have an increased risk of developing hypersensitivity reactions when treated with thiacetazone [544]. About 75% of the patients admitted to an eye ward in Malawi with Stevens-Johnson syndrome were HIV positive [281].

**HIV and Corneal Transplantation in Africa**

Unfortunately, the different epidemiological profile of the AIDS epidemic in Africa [545] and the different circumstances in which keratoplasties are carried out, make screening techniques inadequate for developing countries. In Rwanda, as in most developing countries, there is no eye bank, and donor corneas are used within hours after enucleation, which makes ELISA testing, if available in the first place, difficult [382]. Furthermore the ELISA test can give false-negative results [546]. A simple formula has been derived [546] to calculate the chances of a patient undergoing keratoplasty receiving a cornea from an HIV infected donor despite a negative ELISA. It is 1.2% if the HIV seroprevalence in the donor population is 10% which is realistic for the urban population in certain African countries [254].

**Ocular manifestations of HIV infection in children**

The range of vertical transmission is from 25% to 40% in developing countries [387]. The higher rate in developing countries may be due to the extra risk associated with HIV transmission through breast feeding, but this has yet to be confirmed [254]. In one study of Rwandese HIV-infected children inflammation or sheathing of the peripheral retinal vessels was noted. CMV retinitis was present in 2% and cotton-wool spots, the most common ocular manifestation of HIV disease in adults, were not observed. The most common external finding was multiple mollusca contagiosum on the eyelids and the face, but this is quite common even in healthy African children. In the HIV-infected children these lesions tended to be larger with
a tendency for bacterial super-infection. Other findings included 2/162 cases of herpes zoster, 2/162 cases of conjunctival xerosis that responded to vitamin A administration, and a single case each of conjunctival telangiectasis and recurrent palpebral abscesses. Decreased tear production was also a prominent finding [254].
Ocular manifestations of HIV-1 infection in a rural population based in Uganda. A population based study.

Introduction

The purpose of this study was to report the ocular findings in HIV- seropositive individuals and HIV- seronegative controls in a population-based cohort in rural Uganda. Although many studies have reported a high prevalence of ocular problems in persons infected with HIV-1 and AIDS these studies were based in hospital or outpatient departments in developed and less developed countries. Furthermore they mainly report the ophthalmological findings in patients with late-stage HIV disease or at post-mortem. Few studies have addressed the burden of eye disease caused by HIV-1 in the community incorporating all stages of the diseases. This is especially important in rural areas of less well-developed countries such as Uganda, where the prevalence of HIV-1 in adults is about 10% and access to ophthalmic clinics and eye care is limited.

The ocular findings in HIV-1 seropositive individuals and HIV- negative controls in a population based cohort in rural Uganda is reported.
Methods

A Natural History Cohort (NHC) was established in rural Southwest Uganda in 1990 and consists of HIV prevalent cases of infection identified as HIV seropositive in 1989/90, HIV incident cases and negative controls. Participants are seen routinely every three months in the study clinic and at other times if they are ill. All clinic staff are unaware of, and are unable to find out the HIV status of the participants. The NHC has been described in detail elsewhere. Participants give informed consent (either written or thumbprint) at enrolment, and the study received ethical approval from the Uganda National Council for Science and Technology.

Ocular examination

All available participants underwent ophthalmological examination over a four week period in March 1996 using a standard protocol. The ophthalmologist was unaware of the HIV status and clinical history of the participants. The visual acuity was measured with and without pinhole correction at 6 meters using a Snellen chart if the patient was able to read and, if not, an “illiterate E” chart was used. The visual fields were then assessed to confrontation, a cover test performed and the extra ocular movements examined. Pupil reactions to light and accommodation were noted, and any pupillary irregularities recorded. Lid positions were examined and then the patients were refracted. Corneal sensation was tested using a cotton wool wisp. After installation of local anaesthesia and fluorescein into the conjunctival sac, examination using a Haag Streit slit lamp, including Goldmann applanation tonometry was performed. The presence of any discharge, corneal staining, including any epithelial defects was then noted. Conjunctival lesions were assessed, and the presence of any anterior chamber activity graded as follows:

Cells per 3 mm high slit beam field: and flare
After the iris was examined, the pupils were dilated, and the lens examined for position and opacities. Fundal and vitreous examinations were carried out, both biomicroscopically with a 90 dioptre lens and by indirect fundoscopy using a 20 dioptre lens.

Vitreous inflammation was graded as follows;

Clear = 0
Mild, few scattered opacities = 1
Moderate scattered opacities, fundal details partially obscured = 2
Many opacities, marked blurring of fundal details = 3
Dense opacities, no view of fundus = 4

All participants found to have ocular lesions requiring treatment were taken to the ophthalmic clinic at the local hospital.

**Data collection and statistical analysis**

All findings were directly entered onto the data collection form. Data were double entered and validated, and analysis was done at the main office approximately 100kms away from the study area. EPI-INFO and STATA (Stata Corp 1995. Stata Statistical Software; Release 4.0 College Station, Tx; Stata Corporation) statistical packages were used. Chi squared ($\chi^2$), or if small number Fisher-Exact, 2 tailed test
to compare binary outcomes in HIV groups. For continuous variables, the comparisons were made using 2-sample t-tests. Linear regression was used to test for an association CD 4+ lymphocyte count adjusting for age.

**CD 4+ lymphocyte counts**

T-cell subset analyses are estimated every six months in HIV positive participants and every year in HIV negative participants by FACSCOUNT (Becton Dickinson). The CD 4+ lymphocyte counts included in this paper are estimates performed within 6 months of the eye examination. If several counts were available, we included the one taken closest to the eye examination.
Results

By the time of this study, 410 individuals had been enrolled in the NHC, of whom 64 had subsequently died and 51 had moved out of the study area. 263 participants (46 prevalent, 65 incident cases and 152 HIV negative) were seen for routine appointments during the quarter in which this study occurred, of whom 238 attended for ophthalmic examination. Thus we saw 91% of available participants: 43 were prevalent cases, 62 were incident cases and 133 were negative controls. The HIV-negative group examined tended to be older than both the prevalent and incident cases. The mean ages (standard deviation (S.D.)) of the prevalent and incident cases and negative controls were 39.5 (12.8), 34.4 (12.9) and 42.5 (15.6) years respectively. However, only 2 (4.7%) prevalent, and 4 (6.5%) incident cases were aged over 60 years compared to 26 (19.5%) of the HIV-negatives.

Eleven participants had AIDS (WHO clinical stage 4) at the time of examination (10% of HIV positive patients) and another 4 developed AIDS within six months of the examination. The mean (S.D.) CD 4+ counts in these two groups were 291 (236) and 270 (236) cells/mm$^3$ respectively. This can be compared with the mean CD4+ counts for the whole sample given in Table 15.

Visual loss rates and causes (with pinhole correction)

One person was found to be blind in one eye (visual acuity (VA) less than 3/60). Eight had bilateral low vision (VA 6/24 to 3/60) (2 prevalent, 1 incident and 5 negative cases). Five patients had uniocular low vision (1 prevalent and 4 negatives). Details of these participants, including the causes of visual impairment are given in Table 16.
Table 15: Number of participants seen by HIV category, gender, age and CD4 lymphocyte counts (cells/mm³)

<table>
<thead>
<tr>
<th>Age in years</th>
<th>Prevalent cases</th>
<th>Incident cases</th>
<th>Negative controls</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>(i) Males</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Less than 40 y</td>
<td>14</td>
<td>14</td>
<td>25</td>
<td>53</td>
</tr>
<tr>
<td>Mean CD4 count</td>
<td>531</td>
<td>618</td>
<td>999</td>
<td>775</td>
</tr>
<tr>
<td>(S.D.)</td>
<td>(357)</td>
<td>(163)</td>
<td>(363)</td>
<td>(382)</td>
</tr>
<tr>
<td>40 – 50 y</td>
<td>12</td>
<td>9</td>
<td>25*</td>
<td>46</td>
</tr>
<tr>
<td>Mean CD4 count</td>
<td>354</td>
<td>462</td>
<td>773</td>
<td>599</td>
</tr>
<tr>
<td>(S.D.)</td>
<td>(253)</td>
<td>(138)</td>
<td>(259)</td>
<td>(302)</td>
</tr>
<tr>
<td>60 y and over</td>
<td>1</td>
<td>2</td>
<td>14</td>
<td>17</td>
</tr>
<tr>
<td>Mean CD4 count</td>
<td>192</td>
<td>486</td>
<td>787</td>
<td>717</td>
</tr>
<tr>
<td>(S.D.)</td>
<td>(386 and 585)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(ii) Females

<table>
<thead>
<tr>
<th>Age in years</th>
<th>Prevalent cases</th>
<th>Incident cases</th>
<th>Negative controls</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Less than 40 y</td>
<td>11</td>
<td>29</td>
<td>42</td>
<td>82</td>
</tr>
<tr>
<td>Mean CD4 count</td>
<td>441</td>
<td>723</td>
<td>1103</td>
<td>880</td>
</tr>
<tr>
<td>(S.D.)</td>
<td>(363)</td>
<td>(356)</td>
<td>(269)</td>
<td>(397)</td>
</tr>
<tr>
<td>40 – 59 y</td>
<td>4</td>
<td>6</td>
<td>15</td>
<td>25</td>
</tr>
<tr>
<td>Mean CD4 count</td>
<td>575</td>
<td>476</td>
<td>924</td>
<td>760</td>
</tr>
<tr>
<td>(S.D.)</td>
<td>(327)</td>
<td>(283)</td>
<td>(287)</td>
<td>(348)</td>
</tr>
<tr>
<td>60 y and over</td>
<td>1</td>
<td>2</td>
<td>12</td>
<td>15</td>
</tr>
<tr>
<td>Mean CD4 count</td>
<td>637</td>
<td>348</td>
<td>811</td>
<td>732</td>
</tr>
<tr>
<td>(S.D.)</td>
<td>(341 and 355)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Total

<table>
<thead>
<tr>
<th></th>
<th>Prevalent cases</th>
<th>Incident cases</th>
<th>Negative controls</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>43</td>
<td>62</td>
<td>133</td>
<td>238</td>
</tr>
<tr>
<td>Mean CD4 count</td>
<td>457</td>
<td>618</td>
<td>943</td>
<td>769</td>
</tr>
<tr>
<td>(S.D.)</td>
<td>(322)</td>
<td>(295)</td>
<td>(313)</td>
<td>(386)</td>
</tr>
</tbody>
</table>

CD4 counts:

<table>
<thead>
<tr>
<th></th>
<th>Prevalent cases</th>
<th>Incident cases</th>
<th>Negative controls</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>500 and over</td>
<td>18</td>
<td>39</td>
<td>125</td>
<td>182</td>
</tr>
<tr>
<td>200 – 499</td>
<td>14</td>
<td>19</td>
<td>6</td>
<td>39</td>
</tr>
<tr>
<td>0 – 199</td>
<td>11</td>
<td>4</td>
<td>0</td>
<td>15</td>
</tr>
</tbody>
</table>

*One person in this category did not have a CD4 count estimated within 6 months of eye examination
S.D. Standard deviation
Table 16: Details of participants with visual impairment (less than 6/18)

<table>
<thead>
<tr>
<th>Age</th>
<th>VA Right / Left</th>
<th>Cause(s) of low vision</th>
<th>CD4 count/mm^3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Prevalent cases:</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(i) 76 years* 6/36 6/36 Bilateral cataracts</td>
<td>621</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(ii) 28 years* 3/36 6/24 High myope with tilted disks and left chorioretinal scar</td>
<td>832</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(iii) 48 years 3/24 6/9 Bilateral cataracts, worse on right</td>
<td>388</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Incident cases:</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(i) 60 years NPL 6/24 Optic atrophy and macular degeneration right eye Cataract left eye</td>
<td>341</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(ii) 46 years* 6/24 6/24 Bilateral panuveitis</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Negative cases:</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(i) 56 years* 6/36 6/36 Bilateral cataracts and macular degeneration</td>
<td>615</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(ii) 77 years* 6/60 3/60 Bilateral cataracts, bilateral peripapillary pigmented retinopathy and bilateral RPE changes around disk</td>
<td>1266</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(iii) 72 years* 6/24 6/24 Bilateral cataracts and bilateral retinal degeneration</td>
<td>310</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(iv) 66 years* 3/36 6/36 Bilateral corneal scar and upper pannus, left asteroid hyalosis</td>
<td>905</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(v) 66 years* 6/36 6/36 Bilateral cataract, left corneal scar and right asteroid hyalosis</td>
<td>966</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(vi) 46 years 3/60 6/12 Right eye amblyopic. Left eye uncorrected myopia and cataract</td>
<td>855</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(vii) 70 years 6/18 3/60 Bilateral cataract, macular degeneration</td>
<td>499</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(viii) 30 years 6/6 3/60 Congenital cataracts and cells/flare anterior chamber</td>
<td>1058</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(ix) 72 years 6/24 6/12 Bilateral cataracts and cells/flare anterior chamber</td>
<td></td>
</tr>
</tbody>
</table>

* Bilateral low vision
NPL: Not perceiving light
RPE: Retinal pigment epithelium
**Refraction**

There was no difference between the HIV-positives and HIV negatives for refraction in either the right or left eye. The refraction for the right eye: HIV positives, mean = -0.21 (SD 0.96), n=104; HIV-negatives, mean=-0.11 (SD=0.65) n=131; p=0.4 (2 sample t-test). Refraction for the left eye: HIV positives, mean -0.30(SD= 1.14), n=102; HIV-negatives, mean=-0.12 (SD=0.64), n= 130; p=0.12 (2-sample t-test).

We also looked for an association between refraction and disease progression as measured by CD 4+ lymphocyte counts in the HIV-positive group. The results showed no linear trend for either the right eye (p=0.87) or the left eye (p=0.69, least squares regression adjusting for age).

Overall the rates of myopia (greater than -0.5D) were 12.0% for the right eye and 9.3% for the left eye. Four HIV positive participants and one HIV negative participant had a myopia of greater than -3.0 in the right eye and 3 and 1 respectively in the left eye.

**Intra-ocular pressure**

The mean intraocular pressure (IOP) in the right eye and the left eye of all groups was 12.6 mm Hg. There was no significant association between IOP and CD4+ count (adjusting for age, right eye p=0.16, left eye p=0.25) or HIV group. Seven participants had an IOP greater than 18 mm Hg (2 prevalent cases, aged 20 and 51 years and 7 negative controls, mean age 70 years).

**Ocular Examination**

In addition conjunctival melanosis was seen in 138 of 185 (75%) participants aged less than 50 years and 50 of 53 (94%) of those aged over 50 years. Lens opacities
were also a frequent finding with 46 (25%) of those aged less than 50 years having some degree of cataract, although few had associated symptoms.

Five participants had suspicious looking melanotic conjunctival lesions, however none of these were confirmed as being neoplastic on conjunctival biopsy and histological examination.

Two patients had bilateral peripapillary optic atrophy. One was an 86 year old incident case, with a CD 4+ of 192 and apart from nuclear sclerotic cataracts and a visual acuity of 6/12 in both eyes he had no other ocular abnormalities. The other person was an HIV-negative 69 year old. He had a visual acuity of 6/6 in both eyes and apart from bilateral nuclear sclerosis and peripapillary atrophy his eye examination was normal. Subsequent syphilis serology of this second case was positive (RPR titer = 4, TPHA = 5120).

The ocular findings seen significantly more frequently in HIV-positive subjects were: punctate epithelial erosions (PEE), which were found in 5 HIV positive cases, and no HIV negative controls (p=0.0016, Fisher-Exact 2-tailed) and keratic precipitates (KP) in the anterior chamber (8 HIV-positive cases, one HIV negative control (p=0.012, Fisher-Exact 2-tailed). Perivasculitis (periarteritis and/ or periphlebitis) was seen significantly more frequently in prevalent as compared to incident cases (p=0.008 Fisher –Exact, two-tailed test), although the overall difference between HIV–positive cases and HIV negative controls for this finding was not statistically significant and the condition was not significantly associated with a CD 4+ lymphocyte count of less than 200 cells/mm³.

Overall, 11 (26%) prevalent cases, 6(10%) incident cases and 8 (6%) HIV negatives had some signs of intraocular inflammation, i.e. either KPs or cells in the anterior chamber or choroiditis, vitreous inflammation, retinitis or perivasculitis of fundal
vessels, (HIV positives compared to negatives $\chi^2 = 5.47$, $p=0.020$). In the HIV-positive group, 5/15 (33%) participants with a CD 4+ count < 200 had signs of intraocular inflammation compared to 12/90 (13%) of those with a CD 4+ count > 200 ($p=0.043$) Fisher-Exact 2-tailed). Five out of the six incident cases with signs of intraocular inflammation had been infected with HIV for more than 5 years. All 3 prevalent cases with KPs in the anterior chamber had other signs of intraocular inflammation. Neither cotton wool spots (CWS) nor cytomegalovirus retinitis (CMV) were seen.

**Examination of participants with AIDS**

Of the 11 participants with AIDS at the time of examination, 7 were prevalent cases of infection of whom two died 1 week and 7 months after the examination, and four were incident cases of infection. Three further prevalent and one incident case developed AIDS within 6 months after the examination of whom one prevalent and the incident case had died by the end of 1996. Four of the 7 prevalent cases seen with AIDS and one of the three who subsequently developed AIDS had evidence of intraocular inflammation. None of the incident cases seen with AIDS had ocular abnormalities, except one 78 year old participant with bilateral cataracts and drusen. A further 5 prevalent and 3 incident cases had CD4+ lymphocyte counts of less than 200 cells/mm³ within 6 months of their ocular examination, but did not have AIDS according to the WHO clinical staging system.

Three of the prevalent cases had abnormalities:

1) the 82 year old man with bilateral peripapillary atrophy described above;

2) a 29 year old with a visual acuity in the right and left eyes of 6/12 and 6/9 respectively and bilateral retinal atrophic and pigment epithelial changes
3) a 53 year old with raised intraocular pressure of 24 mm Hg in the right eye with associated shallowing of the anterior chamber and associated pigmentation of the angle and anterior synechiae formation. He also had anisocoria with sluggish pupil reactions and some cellular activity in the vitreous.

Two of the incident cases with CD 4+ counts less than 200, but not with AIDS had eye signs; A 46 year old female with bilateral purulent conjunctivitis and bilateral anterior chamber activity and one patient with K.P.s.

Eleven of the participants were referred and seen at the local ophthalmic clinic, and a further five had conjunctival biopsy. Another six participants were advised to attend the ophthalmology clinic (transport and costs were provided), but they chose not to.
Discussion

This chapter reports the results of a full ophthalmic examination of a cohort consisting of approximately half HIV-positive and half HIV negative participants. The examiner was unaware of their HIV status, and thus represents an unbiased account of ocular findings in the population in rural Southwest Uganda. For reporting individual ophthalmic features, we separated the HIV-positive patients into prevalent cases, all of whom had been infected for more than six years, and incident cases of infection with estimated dates of seroconversion spread over six years from 1990. This was to distinguish any ophthalmic effects in those with longstanding HIV infection from findings in the incident cases, many of whom had only recently become infected. The greater the degree of immunosuppression in the prevalent cases was indicated by a lower mean CD 4+ lymphocyte count in most age groups.

The HIV negative controls included a greater proportion of people aged over 60 years. This is because older people with HIV infection have a more rapid progression of disease than younger people with HIV disease [549, 550], and so the older age groups have borne the brunt of HIV related deaths. Consequently, the ophthalmic features associated with old age such as cataracts, posterior vitreous detachment, drusen and macular degeneration were seen more commonly in the HIV-negative group.

Five participants had conjunctival lesions that were referred to the local ophthalmic unit for biopsy, but none were found to be neoplastic. HIV infection has been strongly associated with the increase of patients presenting with conjunctival malignancies in Uganda [541, 542], and there are suggestions that HIV infection
results in a more aggressive carcinoma. It is therefore essential that all lesions suspected of being malignant are subjected to biopsy and histology.

Our mean refractive error was considerably lower, and our rates of myopia were also higher than reported in those from a rural population in Malawi. Although we found no difference in the refraction between the HIV positive and negative people, four HIV-positive people had myopia greater than -3.0 dioptres compared with one negative control. Myopia either appearing or rapidly increasing has been reported in HIV positive patients. It is not known whether our patients had noticed a change in their vision.

An unexpected finding was that the mean IOP (12.6 mm Hg) was lower than would be found in European countries. Whilst low mean IOP has been reported from Mongolia (12.7 mm Hg in men and 12.5 mm Hg in women) it is not known if IOP varies from one African population to another. Conversely in Barbados, the mean IOP was found to be 18.7 mm Hg in the population of African origin, 18.2 in mixed race participants and 16.6 in whites. We found no significant association between the CD4+ lymphocyte count and IOP, or between HIV-positive patients and HIV-negative controls.

Studies have reported that HIV retinopathy is less common in AIDS patients in Africa, with only 13% of patients admitted to a large referral hospital in Malawi and 15% of AIDS patients in Kigali, Rwanda having retinopathy on ocular examination. This compares to a study in the U.S., where 43% patients with AIDS had retinopathy on initial examination, although none of the HIV-infected but asymptomatic subjects had retinal findings. We did not see the classical retinopathy described in AIDS but we did find that a greater proportion of the HIV-positive patients had evidence of intraocular inflammation, with 26% of prevalent
cases 10% of incident cases and 6% of HIV-negative participants having either KPs or cells in the anterior chamber, vitreous inflammation, retinitis or perivasculitis of the fundal vessels. Evidence of intraocular inflammation was seen in 33% cases of HIV-positive participants with a CD4+ count of less than 200 cells/mm³. Other studies have reported that the prevalence of the classical HIV related retinopathy increases with increased immunosuppression [547, 556] and we have found that this is true for inflammation in this group of participants.

Perivasculitis of the retinal vessels has been reported previously in patients in Africa with AIDS. In one study, 15% patients with AIDS had perivasculitis as the only ocular abnormality and the authors suggested that perivasculitis should be considered as part of the noninfectious AIDS retinopathy [534]. Perivasculitis has also been found in children with AIDS-related complex (but not AIDS) in Africa [390], suggesting that it might be an early manifestation of HIV-retinopathy. In our study, although the numbers were relatively small, perivasculitis was not seen significantly more frequently in HIV positive patients overall, but it was seen more frequently in prevalent compared to incident cases. However it was not associated with a CD4+ of less than 200 cells/mm³, and whilst this would support the theory that this perivasculitis may be an early sign of HIV retinopathy, it also suggests that it maybe related to period of infection rather than degree of immunosuppression.

Cotton wool spots have been reported in about 50% of patients with AIDS in developed countries [140, 547]. The prevalence of CWS seems to increase with the severity of HIV infection and are associated with a poor prognosis [286, 547]. CWS may only be found after repeated examination due to their transient nature [557]; they usually develop over a few days and then regress over a period of 4-6 weeks. In this study no patient was seen with CWS. This could have been due to the transient
nature of these lesions, but if they do occur with any appreciable frequency in this
population, we should have detected some during this study as we examined 15
participants with CD4+ counts less than 200 cells/mm$^3$ (7 of whom had CD4+
counts of less than 100 cells/mm$^3$).

Herpes Zoster is the most common presenting condition of HIV infection to the
ophthalmologist in Africa \cite{558}. We have seen 34 cases of Herpes Zoster in this
cohort, however none of these involved the ophthalmic division of the trigeminal
nerve. Similarly although choroidal lesions are found in HIV-patients with
tuberculosis in Africa, \cite{559} we did not find any lesions in the three participants
diagnosed as having tuberculosis within three months of their examination, and only
one of the participants with intraocular inflammation developed tuberculosis within
9 months after the eye examination (data not presented). Thus although the ocular
complications of herpes zoster and tuberculosis may be seen at ophthalmic clinics,
they do not appear to be common causes of pathology in the rural population. CMV
retinitis has been reported in up to 46% of patients with AIDS in developed
countries \cite{140,286}. No participant in our study had evidence of ocular CMV. By the
end of 1996, we had accrued 375 person years (PY) of observation for the prevalent
cases and 304 PY of observation for the incident cases, and 63 deaths of HIV
positive participants. During this follow up no participant has ever complained of
loss of vision. Other studies have also found that clinical CMV infection is
uncommon in African patients with HIV infection and AIDS \cite{534,560}. Since T-cell
subset estimations were started in the cohort in 1993, 19 people have had CD4+
counts of< 50 cells/mm$^3$ and most survived several months after this reading. So
some HIV infected persons in rural Africa are surviving for an appreciable time with
very low CD4+ lymphocyte counts and low CD4+ count alone does not seem to
account for the occurrence of CMV retinitis. It has been suggested that CWS, which represent a localised disruption of the blood-retinal barrier, act as an entrance port for the CMV [125].

We have found an increasing prevalence of intraocular inflammation in the HIV-positives compared to the HIV negative controls. However, although 43% of HIV positive cases with a CD4+ lymphocyte count less than 200 cells/mm$^3$ and 14% of those with a CD4+ count greater than 200 cells/mm$^3$ had signs of intraocular inflammation, only one person had low vision that could be attributed to HIV. The others reported no ocular symptoms and none had attended the ophthalmology clinic 15 miles away. Thus although the complications of AIDS seem to comprise a large extra element in the work load of tertiary care hospitals dealing with eye problems, the disease does not appear to increase the workload of rural eye care programmes.
Chapter 5 General Discussion and Conclusions

Ocular toxoplasmosis, a retinochoroiditis, is usually regarded as being the result of reactivation of the congenital retinal lesion. Diagnosis, critical for the timely intervention of antiparasitic treatment, is presumptive, based primarily on the recognition of the focal necrotising lesion. However, on occasion the associated vitritis may be so dense as to preclude adequate fundoscopy, thus making diagnosis difficult, furthermore, in several patient groups lesions may be atypical;

Firstly, in the immunocompromised the presenting features of toxoplasma retinochoroiditis may be markedly atypical, with bilateral and multifocal lesions or extensive areas of retinitis occurring. Multiple pathology may also confuse the clinical picture. Typically the lesions may be fulminant and highly destructive.

Secondly, the elderly, who may be more susceptible to severe ocular Toxoplasma infection because of age-related decline in cell-mediated immunity may also present with unusual lesions, which initially may be misdiagnosed as both cytomegalovirus and ARN [106, 561].

Thirdly, ocular disease, associated with both the acute and chronic phase of systemic toxoplasmosis, is now thought to be more common than previously thought [78, 79, 83, 562]. Such disease is not associated with pre-existing choroidal scars; furthermore recurrence of ocular disease associated with acute toxoplasmosis occurs and may arise de-novo in previously healthy retina [563]. Retinal vasculitis and associated intraocular inflammatory reactions without focal necrotising retinitis may be the only ophthalmic manifestations during the early stages of acquired systemic toxoplasmosis [564].
Figure 5-1; Retinitis caused by different Pathogens

Active Toxoplasma retinochoroiditis adjacent to a scar with overlying vitritis

Active peripapillary toxoplasmosis without previous scar

Punctate outer retinal necrosis  CMV retinitis
Under the circumstances where the aetiological agent causing either severe inflammatory disease or an atypical picture, the use of molecular biology as a diagnostic aid becomes increasingly important.

Part of this work concerns the use of the polymerase chain reaction in the diagnosis of ocular toxoplasmosis by the amplification of \textit{T. gondii} DNA from ocular samples taken from patients with active retinochoroiditis. From our initial work we concluded that the amplification of the \textit{T. gondii} B1 gene would provide the most sensitive assay. When the technique was applied to aqueous samples into which known quantities of \textit{T. gondii} tachyzoites had been added, sensitivity was found to be improved by an additional DNA extraction step prior to the subsequent PCR. More complex extraction procedures conferred no advantage to simple heat extraction techniques. When aqueous samples taken from patients with active disease were subsequently subjected to PCR we were unable to amplify target DNA sequences. Controls confirmed that the sensitivity of the reaction was able to amplify as little as 50-100 fg of \textit{T. gondii} DNA. Even when the samples were further diluted to overcome any product inhibition by the sample we were still unable to amplify DNA from aqueous samples. The technique was able to amplify DNA from one of two vitreous samples and from deparaffinised retinal sections.

Although the number of patient samples used in this work was relatively few, we have concluded that aqueous humour is not the most appropriate ocular fluid from which \textit{T. gondii} DNA can be amplified. This would tend to reflect the current published results which have been highly variable in their ability to amplify the DNA from aqueous. Garweg in particular concludes that PCR examination of aqueous samples is insufficiently sensitive to establish the diagnosis in patients with Toxoplasma retinochoroiditis [447, 565].
Why is it that there is such variable results using PCR analysis of ocular fluids? Recent work suggests that in patients with acute acquired toxoplasmosis PCR analysis of ocular fluids is a more useful diagnostic tool than in patients with recurrent ocular toxoplasmosis. The presence of DNA in patients with primary ocular toxoplasmosis and serological evidence of acute systemic toxoplasmosis is possibly caused by the immaturity of the intraocular *T. gondii*-specific immune response, which is not yet capable of clearing the infectious agent in the early stages of the disease [562].

In cases of presumed chronic recurrent ocular toxoplasmosis, most ocular fluid samples have been found to contain antibodies specific to the GRA-2 which is expressed in both the tachyzoite and bradyzoite stages of the parasite [80]. It could be hypothesised that during an initial episode of infection the intraocular immune system becomes primed to tachyzoite antigens. Subsequent episodes of intraocular inflammation, which is thought to be caused by reactivation of tissue cysts within the retina, could be in response to antigens common to both the bradyzoite and tachyzoite stages of the parasite that have leaked from the retinal cysts. Thus the presence of tachyzoites away from the site of reactivation is not a prerequisite for an inflammatory response within the eye. Perhaps in the subgroup of patients with acquired ocular toxoplasmosis PCR analysis of aqueous may be of value in determining the underlying aetiology of disease. In those cases where intraocular inflammation is associated with recurrent reactivation juxtaposed to a retinochoroidal scar, PCR analysis of aqueous appears to be of less use. Under such circumstances the presence of parasite DNA within the anterior chamber might be expected to be uncommon. It is possible that the PCR analysis of vitreous samples may be more useful in the amplification of *T. gondii* DNA [566]. It is perhaps more
understandable that *T. gondii* DNA might be shed into the vitreous during an episode of disease reactivation. Additionally, recent work has suggested that in patients with recurrence of active retinochoroiditis, those that are PCR positive for *T. gondii* DNA in aqueous are also PCR positive for *T. gondii* DNA in blood [567]. Thus in patients with inflammatory eye disease suspected to be related to retinochoroiditis, PCR analysis of blood should be undertaken before the more invasive procedure of paracentesis.

In summary therefore, with respect to the laboratory diagnosis of ocular toxoplasmosis, culture of intraocular fluids is particularly insensitive when only a small amount of fluid is available [568], and it may take days to weeks to obtain a result. The recognition of trophozoites in histological sections can be difficult [217], but can be facilitated by using Giemsa or Wright stains.

The presence of anti-*Toxoplasma* antibodies in the peripheral blood has a low specific diagnostic value in ocular disease [189] due to their high prevalence in the general population. The absence of circulating antibodies on the other hand makes the diagnosis unlikely [188]. Serological diagnosis of *T.gondii* infection can be difficult in AIDS patients. Unlike immunosuppressed cancer patients who consistently have high and diagnostically rising antibody titres when infected with *T.gondii* [91], AIDS patients may not have diagnostically significant serologic findings [134].

Detection of locally produced antibodies may be of some use in immunocompetent patients [187], but is less useful in the immunocompromised [450]. Immunoglobulin G is the major class of intraocular antibody involved in the humoral response to *T. gondii* followed by IgA [215]. Intraocular production of IgG is seen more frequently in patients with recurrent rather than primary ocular toxoplasmosis [562]. There are
marked differences in anti-\textit{T. gondii} IgG specificity in ocular fluid compared to serum in these patients. These differences are not noted in patients with ocular disease associated with recently acquired disease \cite{80}. The coefficient may take some time to become positive in eyes with active disease \cite{210}. A major disadvantage is the occurrence of false negative results in the presence of breakdown of the blood aqueous barrier \cite{187}.

PCR analysis of vitreous appears to be more sensitive than aqueous, although in that group of patients suspected of having active ocular disease associated with acquired toxoplasmosis, PCR analysis of aqueous may be more sensitive than in patients with active recurrent disease. At the present time it seems the diagnosis of Toxoplasma retinochoroiditis remains essentially a clinical one. If the picture is complex certain clinical features may help to establish the diagnosis, but ultimately the response of the disease to a therapeutic trial may be the only way to establish the nature of the causitive agent of disease \cite{106}.

Treatment with HAART has been shown to be accompanied by a decrease in HIV viral load and increase in CD4+ count \cite{517,569-572}.

Before the introduction of HAART up to 40% of HIV-infected patients developed CMV retinitis, the risk being greatest when the CD4+ cell count fell below 50 cells/\mu l \cite{333}. The effect of potent antiretroviral therapy on the immunologic status of patients with human immunodeficiency virus disease has changed the manifestation and course of CMV retinitis in many patients. The clinical management of CMV retinitis is thus changing. Since its first association with HIV/AIDS, cytomegalovirus retinitis has gone from being a pre-morbid diagnosis to one where patients are surviving for increasing lengths of time. The use of HAART therapy,
particularly the introduction of the proteinase inhibitor group of drugs has allowed this group of patients to have a much improved life expectancy.

A longitudinal study of patients with CMVR was undertaken to assess the effect of highly active antiretroviral therapy (HAART) including HIV proteinase inhibitors (PIs) on the survival of patients with cytomegalovirus retinitis (CMVR).

Multivariate analysis showed that the strongest independent predictor of improved survival was having ever received a PI after the diagnosis of CMV retinitis (relative risk of death = 0.063; 95% CI = 0.027-0.149; p<0.0001). The mean survival being 914 days in those who took a PI (n= 24; 95% CI 768-1059 days; 1 year survival 83%; p<0.0001). Others have confirmed this increase in survival of patients with CMV retinitis receiving HAART including a PI [570].

Under such circumstances quality of life issues are becoming an increasingly important part of patient management. The long-term natural history of the disease in patients treated with this group of drugs, however, remains unknown, and may well change if viral resistance to HAART starts to emerge.

The incidence of newly diagnosed CMV retinitis appears to be decreasing [572]. In HIV positive patients with a CD4 cell count below 100 cells/µl the cumulative incidence of CMV retinitis has recently been found to be 5% at 1 year and 6% at 2 years [571]. Development of newly diagnosed CMV retinitis seems to occur in those patients who have a very low CD4+ count when HAART therapy is initiated. In these cases development of disease usually occurs within the first 2 months of initiation of HAART therapy, often despite marked rises in CD4+ count. This maybe because specific immunity to CMV takes some time to become re-established [573].

Positive CMV viraemia, as measured by PCR at initiation of HAART therapy,
including a PI, has been found to be significantly associated with the development of
disease \[571\].

Others have shown that in the absence of specific anti-CMV therapy, HAART
including a PI can not only result in the complete suppression of CMV viraemia
\[574\], but also in those patients who have an increase in CD4+ count in response to
therapy, complete regression of CMV retinitis \[575, 576\].

Relapses of CMV retinitis appear to be less frequent \[517, 572\] but they can occur, at
least in the initial period of treatment \[570, 577-579\] and may occur in patients with
CD4+ count of > 50 cells/µl \[580\]. The sub-group of patients who have poor initial
response to HAART therapy (as measured by CD4+ count) appear to be susceptible
to disease relapse \[577\].

Although recently developed antiviral and immune based therapies have become
available that can dramatically increase the number of peripheral blood CD4+ cells
of HIV-infected individuals to normal or near normal levels, it remains uncertain
whether these therapies will result in immune restoration. Recent studies of subjects
infected with HIV have produced conflicting results about the extent of
reconstitution possible in the CD4+ repertoire after HAART. Using flow cytometry
to study CMV-specific CD4+ lymphocyte responses Komanduri et al demonstrated
strong CMV-specific CD4+ lymphocyte responses in HIV-infected patients treated
with HAART either if the patient had no history of CMV associated end organ
disease, or in most patients treated with HAART and ganciclovir with quiescent
CMV disease. The presence of active end organ disease strongly correlated with loss
of CMV-specific lymphocyte responses \[581\]. Connors et al found in their study of
three patients receiving interleukin 2 and antiretroviral therapy (including indinavir)
that disrupted CD4+ repertoires are not restored after therapy. They go onto urge
caution when considering discontinuation of prophylaxis in the context of rapid increases in CD4+ cell count [513]. Others have found that HAART causes an early rise in memory CD4+ cells, an improved CD4+ reactivity to recall antigens, and a late rise in "naïve" CD4+ lymphocytes and concluded that decrease in the HIV load can reverse HIV driven activation and CD4+ cell defects in advanced HIV-infected patients [582]. Gorochov et al found normalization of CD4+ repertoire in most patients treated with HAART, however some patients did not respond to triple therapy and the CD4+ repertoire was not normalized in such patients [583].

Li et al concluded that HAART can induce sustained recovery of CD4 T-cell reactivity against opportunistic pathogens in severely immunosuppressed patients. This recovery depends not on baseline values but on the amplitude and duration of viral-load reduction and the increase of memory CD4 T cells [569].

The question remains is it safe to discontinue anti-CMV maintenance therapy in patients with quiescent CMV retinitis and increased CD4+ counts after treatment with HAART? Whitcup et al concluded that maintenance anti-CMV medications were safely stopped in those patients who had stable CMV retinitis and elevated CD4+ cell counts and who were taking HAART [584]. Jabs et al concluded that in selected patients with immune reconstitution after initiation of highly active antiretroviral therapy that anti-cytomegalovirus therapy may be safely discontinued, at least temporarily. He suggested that a reasonable guideline for the interruption of anti-CMV maintenance therapy to be:

A rise in CD 4+ T-cell count to > 100 cells/μl; the rise must be by at least 50 cells/μl; and must be sustained for a minimum of 4 months prior to cessation of anti-CMV therapy.

Vrabec et al came to similar conclusions [585].
Jabs et al added that longer follow-up of these patients is needed to determine how long such therapy may be interrupted, and when anti-cytomegalovirus therapy should be reinstituted [586].

Although the incidence of newly diagnosed CMV retinitis has fallen dramatically, patients treated with HAART still require ocular follow-up because increased CD4+ cell counts and decreased viral loads do not always fully protect from active disease [572]. There is also increasing frequency of HIV resistance to highly active antiretroviral therapy [587-589].

At the end of 1998, an estimated 67% of people living with HIV/AIDS in the world were in Sub-Saharan Africa—some 22.5 million people [590]. In contrast to the Western world where the use of HAART has increased life expectancy, in this region life expectancy has been reduced by one quarter [591]. In only two countries, Uganda and Senegal, does the epidemic seem to be abating. Here strong governmental leadership ensures that there is universal health education, that condoms are easily available, and that there is coordinated action from the government [591]. In such resource-poor areas the challenges of disease management and patient care are very different from those in the Western world; here prevention must take priority [592]. Sub-Saharan Africa has been particularly hard hit by the disease because of a combination of poor social and health education and also because of other endemic sexually transmitted diseases which significantly increase the chance of HIV transmission [593]. The most important challenge in this region therefore is to reduce the number of new infections: simple interventions such as barrier methods
of contraception and better treatment of sexually transmitted diseases must be implemented now whilst waiting for an effective vaccine [590].

Few studies have addressed the burden of eye disease caused by HIV-1 in the community incorporating all stages of the diseases. We report the ocular findings in HIV- seropositive individuals and HIV- seronegative controls in a population-based cohort in rural Uganda.

In conclusion this thesis has investigated the use of PCR amplification of T. gondii DNA as an aid in the diagnosis of T. gondii retinochoroiditis. Ocular samples taken from patients with active retinochoroiditis were examined with this technique following careful and systematic laboratory work to maximise the reaction sensitivity, firstly in sterile water and then in both non-inflamed and inflammed aqueous. The effect of sample dilution was investigated to ensure that false negative results did not occur as a result of aqueous inhibition of the PCR reaction, and on the basis of experimental evidence a simple DNA extraction step was included to further enhance reaction sensitivity. Despite this we were unable to amplify DNA from aqueous samples, but were able to demonstrate the appropriate PCR product in vitreous samples from patients with active retinochoroiditis and from retinal tissue containing cysts. It is concluded that vitreous rather than aqueous is probably a more reliable source of parasite DNA.

Also presented herein is a longitudinal study of patient survival with CMV retinitis following the introduction of HAART. This has shown that the use of such treatment has resulted in the increasing survival of this group of patients especially if such a treatment regimen contained a proteinase inhibitor.

Finally, although HIV infection presents a huge health care problem in Uganda, it does not seem to present a great burden to rural eye clinics.
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