TOXOPLASMA GONDII:

AN INVESTIGATION OF INFECTION IN THE IMMUNOCOMPROMISED HOST

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ABSTRACT

The aim of this study was to develop a sensitive and specific method of detecting *Toxoplasma gondii* in the immunocompromised host which would reduce the need for other tests and would ensure the prompt initiation of the appropriate treatment, the effects of which could be monitored. Such a system would also be of benefit in the investigation of parasite / host interaction.

Initial work investigated an antigen ELISA and the PCR using two different gene targets (B1 and P30) to find the most sensitive system. The ELISA was insensitive but both PCR systems were capable of detecting parasite in blood, lymph and tissue samples from experimentally infected sheep. The B1 PCR detected parasite earlier and over a significantly longer period than the P30 PCR, this greater sensitivity being due to the higher copy number of the B1 gene.

The PCR was applied to samples from patients with AIDS with the aim of finding an ideal sample for the diagnosis of infection. Parasite was detected in blood up to a month prior to clinical signs of infection, and therefore blood samples are ideal for monitoring patients at risk of recrudescence of a chronic infection. This result indicates that recrudescence is not due to local reactivation, but is due to a more widespread parasitaemia. However, as parasitaemia was shown to be transient in cases of recrudescence, sampling time may be critical. Parasite was also detected in urine, biopsy tissue and post mortem material, but was not detected in CSF.

Dexamethasone was used to create a mouse model of recrudescence in the immunocompromised patient to further investigate interaction between the parasite and host. The PCR detected parasite in blood, brain and heart of chronically infected animals, however the detection rate was significantly higher in groups receiving immunosuppressive therapy. Dexamethasone treatment mimicked the effects seen in the AIDS population where 30-35% of chronically infected individuals showed clinical signs of toxoplasmosis. However the PCR may also be detecting latent cysts in tissue samples, and blood samples were occasionally positive without clinical evidence of infection. This could be due to small amounts of parasite circulating intermittently, or to breakdown products from parasite degradation.

There was therefore a need to differentiate between active and chronic infection, and this was carried out by developing a quantitative PCR based on competitive amplification. A novel Sma I restriction site was created within the P30 gene, and known amounts were co-amplified with samples. The amplified products were then digested with Sma I to differentiate between mutated and *T. gondii* DNA and the point at which product yield was equalled indicated the amount of original DNA present in the sample. The system was shown to work using human PM samples, and could be adapted to indicate a cut-off point where parasite DNA levels reveal active infection.

In conclusion the B1 PCR is the method of choice in detecting *T. gondii* in AIDS patients. Any patient in which active parasite is detected should be treated and closely monitored using the qPCR for any evidence of reactivation.

DECLARATION

I hereby declare that I alone have written this thesis and that, except where so stated, the work presented is my own.

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ABBREVIATIONS

ABELISA	avidin-biotin enzyme-linked immmunosobent assay
AIDS	acquired immune deficiency syndrome
APS	ammonium persulphate
Amp	ampicillin
bp	base pair(s)
BSA	bovine serum albumin
cag	circulating antigen
CAT	computer axial tomography
CD4+	cells bearing the CD4 antigen
cDNA	complementary DNA
CFT	complement fixation test
CIC	circulating immune complexes
CIP	calf intestinal phosphatase
CMV	cytomegalovirus
CNS	central nervous system
CSF	cerebrospinal fluid
DAT	direct agglutination test
ddNTP	dideoxynucleoside 5'-triphosphate
DIG	digoxigenin
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside 5'-triphosphate
DT	dye test
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
f.u.	follow-up
HE	haematoxylin-eosin
HIV	human immunodeficiency virus

HRP	horseradish peroxidase
IFAT	immunofluorescent antibody test
IHA	indirect haemagglutination
Ig	immunoglobulin
i.p	intra-peritoneal
IPTG	isopropyl-ß-D-thiogalactopyranoside
KB	kilobase pairs
KDa	kilo dalton
LAT	latex agglutination test
L-broth	Lauria broth
MCS	multiple cloning site
MI	mouse inoculation
MRI	magnetic resonance imaging
OPD	O-Phenylenediamine
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBST	PBS containing 0.05% Tween 20
PCR	polymerase chain reaction
PEG	polyethylene glycol
p.i.	post infection
PM	post mortem
qPCR	quantitative PCR
RBC	red blood cell
rDNA	ribosomal DNA
RNA	ribonucleic acid
rRNA	ribosomal RNA
SDS	sodium dodecyl sulphate
srDNA	subunit ribosomal DNA
ssDNA	single-stranded DNA
TE	tris EDTA

TEMED	N,N,N',N', tetramethylethylenediamine
Tet	tetracycline
tris	tris (hydroxymethyl) methylamine
TSS	transport and storage solution
UV	ultraviolet
v/v	volume / volume
WB	whole blood
WBC	white blood cell
w/v	weight / volume
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-thiogalactopyranoside

CHAPTER 1

Introduction

1.1 BACKGROUND

Interest in *Toxoplasma gondii* has increased greatly since its discovery in 1908 by Nicolle and Manceaux (cited by Fleck 1989b). The parasite's ability to infect a wide range of warm blooded animals has implications for both veterinary and human medicine. Infection in animals and humans is generally inapparent or mild, but infection during pregnancy can result in both high morbidity and mortality of the foetus. There are also serious implications for immunocompromised patients who have increased in numbers in recent years due to drug therapy, transplantation and the acquired immune deficiency syndrome (AIDS). It is this relatively new problem which has resulted in a substantial increase in research into the parasite and its complex lifecycle.

1.2 TAXONOMY

The name *Toxoplasma gondii* is derived from the appearance of the parasite and from the rodent *Ctenodactylus gundi*, from which it was first isolated. The genus name is from toxon, Greek for bow, and refers to the crescent shape of the tachyzoite. The full classification can be seen in Figure 1.1 as described by Wong and Remington (1993).

Figure 1.1 Classification of *T. gondii*

Phylum:	Apicomplexa
Class:	Sporozoa
Subclass:	Coccidia
Genus:	Toxoplasma
Species:	gondii

1.3 HISTORY

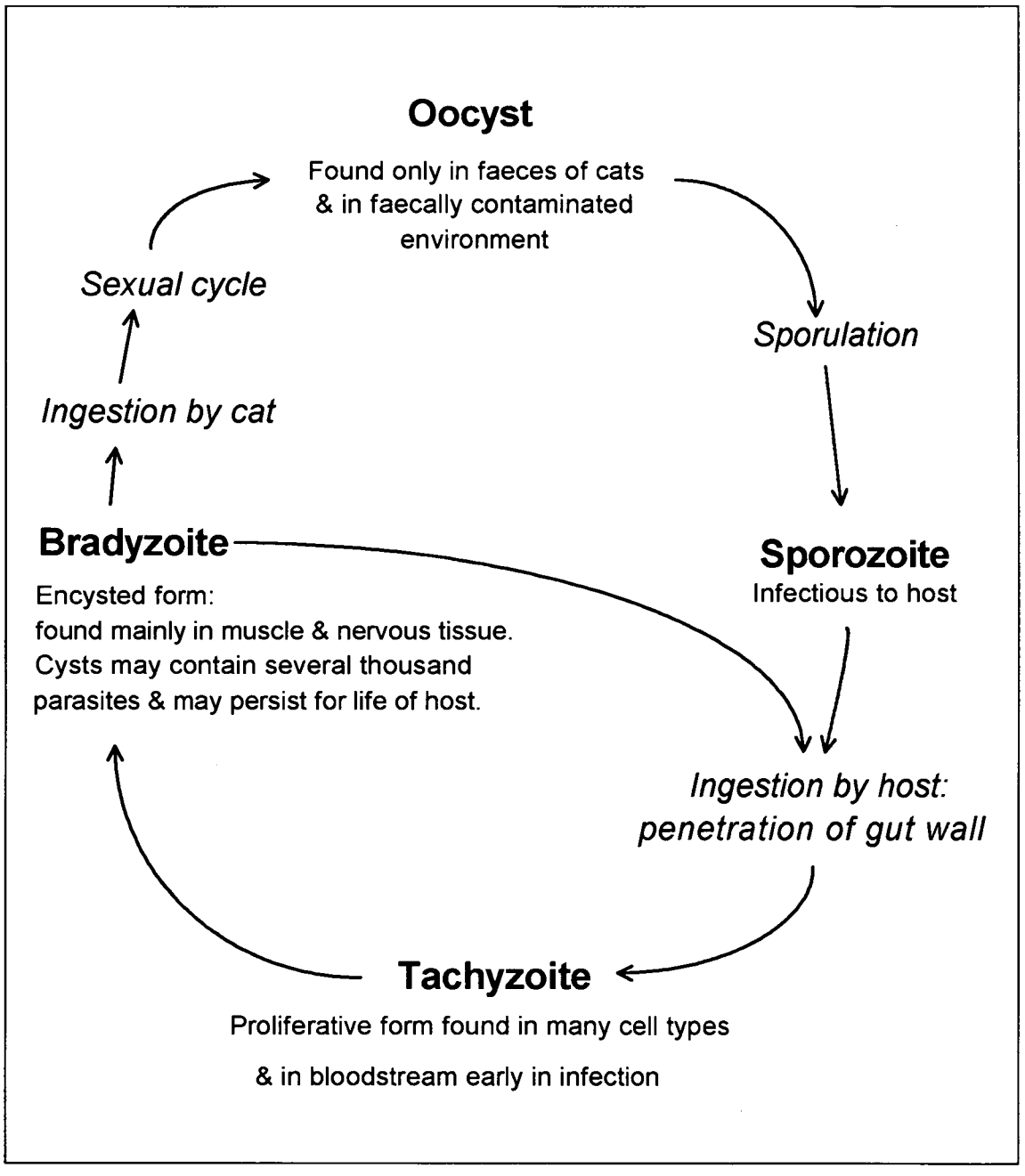
The parasite was first recorded at the beginning of the century and in 1923 cysts were found in the retina of an infant (described by Dutton 1989). In 1937 Wolf and Cowen reported cytoplasmic bodies in a case of megalencephaly. Sabin (1942) first suggested that these were obligate intracellular parasites, and demonstrated the importance of congenital infection and described signs of infection such as microcephaly, chorioretinitis, retinal calcification and convulsions. The connection between the consumption of undercooked pork and toxoplasmosis in humans was described by Weinman and Chandler in 1956, and cysts in meat from cattle, sheep and swine were first described by Jacobs *et al.* (1960b). However the discovery of the parasite in cat faeces by Hutchison (1965) was the key to discovering more about the complex lifecycle involved in the transmission of the disease.

1.4 LIFECYCLE

1.4.1 The Sexual Cycle

The sexual cycle of *T. gondii* can only occur in the cat family which is the definitive host (Figure 1.2). Kittens usually become infected as they first start to hunt and consume meat containing tissue cysts. These cysts disrupt to release bradyzoites which differentiate into micro- and macro-gametes in the epithelial cells of the intestine. Fusion of these gametes results in the formation of a zygote which is surrounded by a protective wall and is known as the oocyst. These are shed in the faeces, and can be detected three days after ingestion of tissue cysts, with up to ten million being produced every day over a period of one to two weeks (Frenkel and Dubey 1972). Oocysts are not infectious until meiosis and sporulation occur between one and five days after shedding in the faeces. Oocysts containing eight sporozoites can remain viable in the soil for a period of months to years depending on conditions, with high moisture levels and a lack of frost increasing survival time (Ruiz *et al.* 1973).

Figure 1.2 The lifecycle of *T. gondii*



1.4.2 The Asexual Cycle

When ingested by a susceptible intermediate host, the infectious sporozoites invade the cells of the digestive system and form tachyzoites, the proliferative form of the parasite. These are 1.5 μm wide and between 3 and 4 μm long (Fleck 1989a). The tachyzoites may be detected in the bloodstream early in infection, and invade most tissues of the body. The infection becomes latent in the tissue cyst form, which contains bradyzoites, and may persist for the life of the host. The lifecycle is maintained either by the ingestion of the tissue cysts by susceptible cats and the sexual cycle, or by the asexual cycle with ingestion of infected meat by an intermediate host.

1.5 TRANSMISSION AND CELL INVASION

Jacobs *et al.* (1960a) suggested that the consumption of raw or undercooked meat was responsible for transmission of *T. gondii* after the discovery that tissue cysts could survive the digestive process. In addition the group recognised that another route was indicated when strict vegetarians were also shown to have antibody and by 1970, infection through feline faecal contamination was seen to provide a further means of transmission (Sheffield and Melton 1970). Frenkel *et al.* (1970) provided support with the discovery that oocysts were capable of infecting mice after four months in soil. Oral infection with oocysts, either directly or via transport hosts such as flies is a means of transmission to the non-carnivorous host (Miller *et al.* 1972). Cat litter trays (Frenkel and Dubey 1972), and contaminated drinking water (Benenson *et al.* 1982) have been demonstrated to be sources of infection. *Toxoplasma gondii* in the human population can also be transmitted vertically to the foetus (Desmonts *et al.* 1985), and through medical procedures such as organ transplantation (Luft *et al.* 1983a), blood transfusion (Siegel *et al.* 1971) and accidental inoculation. These risks will be discussed further in section 1.8.

The observation that *Toxoplasma* can invade almost all cells, including non-phagocytic cells, stimulated a debate on the methods used by the parasite to enter host cells. Nichols and O'Connor (1981) found that cell invasion was more likely to occur by active penetration by the tachyzoite, whereas phagocytosis of the parasite occurred only rarely.

A study by Werk (1985) demonstrated that cell invasion by the tachyzoite occurs within 15 to 40 seconds. In 1991 Achbarou *et al.* suggested that microneme proteins may contain receptors which are involved in parasite / host cell interaction, an area which deserved more investigation. Ossorio *et al.* (1992) found that ROP1, a rhoptry protein plays a part in host cell penetration.

Immunity to the parasite is largely cell mediated although specific antibodies do have a role to play (Buxton 1993). Wilson and Remington (1979) found that phagocytes restrict rather than aid the initial dissemination of *T. gondii*. Interferon γ and other lymphokines are produced by sensitised T lymphocytes and an oxidative burst, produced by mononuclear phagocytes, kills the majority of organisms (Suzuki *et al.* 1991). Makioka and Kobayashi (1991) found that the recombinant P30 antigen (surface membrane protein of the tachyzoite) produced a fusion protein and was capable of activating macrophages to kill *T. gondii in-vitro.* As replication of the parasite becomes inhibited, cyst formation occurs in tissues throughout the host.

A marked difference in susceptibility of inbred strains of mice to *T. gondii* was described by McLeod *et al.* (1989) who found that the degree of tissue infection after oral inoculation was controlled by at least five genes, primarily by the H-2 complex. This has raised the possibility that host genetic differences may contribute to the development and severity of the illness. Suzuki *et al.* (1991) found similar results in toxoplasma encephalitis after intra-peritoneal (i.p) inoculation of mice.

1.6 MOLECULAR ANALYSIS

T. gondii has been established as ancient from studies involving sequencing of the small subunit ribosomal RNA (srRNA) (Johnson and Baverstock 1989). This technique was used to provide information on the phylogenetic relationships of parasitic organisms based on the nucleotide divergence for 215 semi-conserved sites of the srRNA gene. The study revealed that the development of *T. gondii* is genetically closer to *Sarcocystis* than *Plasmodium.*

Little is known about the genetic background which regulates transition between the various forms of the parasite. However pulse field electrophoresis has revealed at least eight chromosomes (McLeod *et al.* 1991). The nucleus has been shown to be haploid during most stages of the lifecycle. However after the sexual cycle the diploid zygote is formed in the feline intestine as the unsporulated oocyst. The genome is estimated to consist of 8×10^7 base pairs (bp) with 53% (Neimark and Blaker 1967) or 55% (Cornelissen *et al.* 1984) guanine and cytosine content.

The several genes which have been identified and sequenced include the P30 gene which encodes for the P30 surface protein (Burg *et al.* 1988), the α and ß tubulin genes (Nagel and Boothroyd 1988), and the P23 (Cesbron-Delauw *et al.* 1989), P28 (Prince *et al.* 1989), and P22 (Prince *et al.* 1990, Parmley *et al.* 1992a) genes. While these genes are known to have a clear purpose, others have also been investigated, including the B1 gene (Burg *et al.* 1989), but their functions are still unclear. Although the P30 gene contains 5 tandem repeats, only the B1 gene is tandemly reiterated and contains between 25 and 50 copies depending on the strain. This 2.2 kilobase (Kb) gene was singled out by McLeod *et al.* (1991) as a potential target for gene amplification for use as a diagnostic tool.

1.7 ANIMAL INFECTION

Primary *T. gondii* infection in ewes during pregnancy results in significant losses to the sheep industry due to abortion or still birth (Buxton 1990, Dubey and Kirkbride 1989). It is also a significant problem in goats and pigs (Weinman and Chalmer 1956). Tissue cysts have been located in lamb (Dubey and Kirkbride 1989), pork (Dubey *et al.* 1986) and swine (Jacobs *et al.* 1960b). An accurate assessment of the scale of infection in farm animals cannot be made due to the prohibitive cost of screening large numbers for evidence of *Toxoplasma* IgG. Other factors such as the rapid loss of *Toxoplasma* antibody in cattle after infection make serological surveys of limited value in these animals. In the UK, toxoplasmosis is a problem in 10-20% of flocks. However the incidence of clinical toxoplasmosis in breeding ewes in Britain is estimated to be 2.2% per annum (Blewett and Trees 1987). The lack of a fully developed immune system

leaves the foetus poorly defended, and infection can lead to death particularly if it occurs early in gestation (Buxton and Finlayson 1986). Results from a study by Dubey and Kirkbride (1989) indicate that epizootics of *Toxoplasma* may occur in congenitally infected lambs. The most commonly recognised source of infection is contaminated pasture (Buxton 1990).

Drugs such as monensin, an ionophorous antibiotic, have been used to reduce losses during lambing in experimental infections with *T. gondii* (Buxton *et al.* 1988). However, the production of a suitable vaccine would be more appropriate in the prevention of infection in sheep and other domestic animals.

1.7.1 Vaccines

Initial studies explored the possibility of passive infection using anti-tachyzoite serum or active immunisation with an experimental killed vaccine (Krahenbuhl *et al.* 1972) to induce protection against acute *Toxoplasma* infections. Partial protection was achieved with these methods.

Later, purified antigens such as P22 and P30 were investigated. Kasper *et al.* (1985) found that these purified membrane proteins resulted in a high mortality rate in mice later challenged with parasite. However, Bulow and Boothroyd (1991) later found that immunisation with P30 in liposomes did successfully protect mice against challenge with *T. gondii*. Results revealed that only one out of 15 vaccinated mice died after subsequent exposure to the parasite compared with 11 out of 15 control mice. They suggested that the type of adjuvant and the route of administration were important in the success of the vaccine. Liposomes are a suitable adjuvant for use with P30 as they possess a hydrophobic lipid anchor, and they are also chemically stable. This study also found that P30 is highly conserved between strains and therefore appropriate for development into a subunit vaccine.

Work has been carried out on a vaccine using live S48 tachyzoites to protect ewes (O'Connell *et al.* 1988, Wilkins *et al.* 1988, Buxton *et al.* 1991, Buxton 1993).

Vaccinated animals produced a higher number of viable lambs compared to non-vaccinated animals in experimentally infected pregnant ewes (72% compared to 17.8%). This strain has an incomplete lifecycle and so does not encyst. Therefore it poses little public health risk from the consumption of meat from inoculated animals.

1.8 HUMAN INFECTION

Toxoplasma gondii is one of the most common protozoan infections of man, affecting approximately 500 million people world wide (Hughes 1985). Levels of infection vary greatly from 80% in France, to 40 - 50% in the US (Wanke *et al.* 1987), and 20% in Scotland (Williams *et al.* 1981). The prevalence of infection would appear to be related to eating habits, climate and sanitary conditions. A higher incidence of antibody positive individuals was found in lower socio-economic groups and in those subjected to obvious risk factors such as the consumption of raw or undercooked meat, and exposure to an environment contaminated by feline faeces (Mills 1986). *Toxoplasma* does not transmit directly from one person to another, with the exception of congenital infection, where it can cross the placenta.

1.8.1 Clinical Signs of Infection

The vast majority of individuals infected with *T. gondii* suffer no serious clinical illness with the infection being self limiting and requiring no treatment (Krick and Remington 1978). However infection can result in symptoms as varied as lymphadenopathy, myocarditis, encephalitis, brain lesions and blindness (Holliman 1988, Luft and Hafner 1990). There are three main groups of individuals who are at risk from serious disease which may even result in death: congenital infection when the mother contracts a primary infection during pregnancy (Desmonts *et al.* 1985); those immunosuppressed due to drug therapy for organ transplant or cancer therapy (Derouin *et al.* 1986); those with an immunological deficiency such as in AIDS (Luft and Remington 1988, Holliman 1991).

Congenital infection

Epidemiological studies indicate that the majority of *T. gondii* infections leading to ocular toxoplasmosis occur *in utero* (Perkins 1973). Infants with congenital

toxoplasmosis are asymptomatic at birth or have non-specific symptoms such as jaundice, hepatosplenomegaly and intrauterine growth retardation. Infection in these individuals may only become apparent after up to 20 years (Johnson J. 1992), when sequelae such as retinochorditis occur. A minority do have severe manifestations including the *Toxoplasma* triad of hydrocephalus, intracranial calcification and retinochorditis (Hall 1992). Other complications can involve widespread tissue destruction of the central nervous system (CNS). Organs such as the liver, heart and lungs may also be affected. Maternal parasitaemia leads to congenital transmission in 40-50% of foetuses (Joss *et al.* 1990), with infection during the first trimester causing most damage, although only 17% of cases are acquired during this period (Desmonts and Couvreur 1974). This is almost certainly due to the immature stage of the immune system and the greater effect of lesions on a small embryo, where several organs still in the early stage of development can be affected. As the foetus gets older so the immune system develops and offers more protection which may reduce the severity of infection (Ambroise-Thomas and Pelloux 1993).

The scale of the problem in the UK remains unclear. Joss *et al.* (1990) and Ho-Yen (1990) estimate 2 out of 1000 pregnancies are affected to some degree with an estimated 73 cases of congenital toxoplasmosis in Scotland each year. It was calculated from this that between 50 and 70 neonates will be seriously affected in England and Wales each year (Hall 1992). Fleck (1989a) reported estimates of a minimum of 130 asymptomatic cases a year, 30 with mild to severe symptoms and 15 resulting in neonatal death. At the highest estimate (Fleck 1989a) there may be 1,300 asymptomatic cases, 300 showing mild symptoms, 200 severe symptoms and 150 neonatal deaths per year.

It is generally believed that only primary infection during pregnancy results in congenital infection. However some evidence challenges this belief (Holliman 1994), suggesting that the incidence of congenital toxoplasmosis from chronic maternal infection may occur with an incidence of 0-6%. The increase in mothers with human immunodeficiency virus (HIV) infection and latent toxoplasmosis may therefore lead to recurrent parasitaemia

and to congenital infection although the mother may remain asymptomatic throughout the pregnancy (Holliman 1994).

Management and prevention of infection during pregnancy has become an important issue which has been increasingly covered by the media. Health education remains the primary method of prevention, recommended by Jeannel *et al.* (1990) as vaccines are not yet available for use in women. In the UK there has been debate on the need for a screening programme for pregnant women, however this was rejected by a multidisciplinary working group on the grounds that there was a lack of evidence that benefits would outweigh the risks and costs involved (Hall 1992). The cost of the disease was estimated at £14 million by Beattie (1980) who was not convinced that universal surveillance and treatment would be cost effective. Others such as Ho-Yen (1990) recognise the benefits of a screening programme. This study estimated the cost of three tests throughout pregnancy to be between 0.7 and 1.2 times the preventable cost of providing care and support for affected children. Fleck (1989a) suggested a pilot study to determine whether such screening would be useful in the UK, and also that there should be more discussion between obstetricians, microbiologists and paediatricians to decide the best policy.

Transplant Patients

A study by Luft *et al.* (1983a) showed that a sub-population of patients who had undergone cardiac transplant, and who received immunosuppressive therapy to prevent rejection, developed serological changes consistent with *T. gondii* reactivation. Derouin *et al.* (1986) revealed that the clinical and serological manifestations of *T. gondii* are closely related to the recipients' serological status before transplantation. If the patient has evidence of past infection, immunosuppressive therapy may lead to secondary reactivation. This is generally asymptomatic, or in the form of a mild illness (Holliman 1990). If a susceptible patient receives an organ from a donor with previous *T. gondii* infection there is a high risk of the development of serious or fatal toxoplasmosis (Wreghitt *et al.* 1989, Holliman *et al.* 1990a). As the parasite lies dormant in muscles, any organ from a donor with past infection may contain cysts. These could then

reactivate when the organ is introduced into a naive recipient producing a primary infection (Speirs *et al.* 1988). Evidence of infection transmitted by a donor was found to be greater in cases of cardiac (57%), than in liver transplants (20%). No evidence of donor transmitted infection was found in renal transplants. This may be because *Toxoplasma* cysts are rarely if ever seen in the kidney (Speirs *et al.* 1988).

AIDS Patients

T. gondii is one of the most common and treatable causes of CNS infection in AIDS patients. The incidence of toxoplasmosis in two AIDS surveys was shown to be 13.1% in Spain (Pedrol *et al.* 1990), and 3-10% in the USA (Luft *et al.* 1993). Luft and Remington (1988) reported that 95 % of toxoplasma encephalitis is due to recrudescence of a latent infection in these patients. This usually occurs when their CD4+ cell count drops below 100 mm^{-3} (Katlama 1991). This count is a marker of disease progression as the virus attacks T helper / inducer lymphocytes bearing the CD4 surface antigen. The defective cellular immune response interferes with the ability to mount an adequate response to intracellular organisms such as *T. gondii*. In a study by Porter and Sande (1992) involving 115 patients with AIDS and CNS toxoplasmosis, the mean CD4+ cell count was 50 mm^{-3}. Vollmer *et al.* (1987) used a murine model in which T4 lymphocytes had been depleted and revealed an overwhelming systemic infection in acutely infected mice, with encephalitis in chronically infected mice.

In the USA where 10-40% of those with HIV have positive *T. gondii* serology, one third of these IgG positive patients will develop toxoplasma encephalitis (Luft and Remington 1992). Early estimates were lower at 6-12% (Wong *et al.* 1984). Grant *et al.* (1990) detected antibody to *T. gondii* in 32% of patients, in which CNS toxoplasmosis developed in 24%, and estimated that 26% of patients were likely to suffer recrudescence within two years of the onset of AIDS. It is not clear why only 25-33% of patients with HIV and latent *T. gondii* infection develop toxoplasmosis. Host factors such as genetic disposition, observed by McLeod *et al.* (1989) and Suzuki *et al.* (1991), which was discussed in section 1.5 may account for this, but variation in virulence may also play a part.

In patients with chronic *T. gondii* infection who later develop AIDS the latent parasite can reactivate and invade many tissues. The parasite is believed to invade the CNS resulting in brain lesions and encephalitis. As the parasite can infect any cell in the brain, clinical symptoms of toxoplasma encephalitis are non-specific (Luft *et al.* 1993). Symptoms typically include seizures, headache and an altered mental state (Luft and Remington 1988). The development of infection in nervous tissue may be due to the lack of immunological control in the CNS (Ambroise-Thomas and Pelloux 1993). However the mechanism of parasite release from tissue cysts remains unclear and so the recognition of a system of local or systemic reactivation still requires investigation. Luft and Remington (1992) suggested that haematogenous spread of the organism may account for the multiple areas of the brain which are involved. Lymphadenopathy may be apparent, and other tissues such as the eyes are also affected resulting in chorioretinitis (Holland *et al.* 1988), and in severe cases the infection may become widely disseminated (Jacobs *et al.* 1991) for example causing pneumonitis.

1.8.2 Diagnosis

Direct methods of diagnosis of *T. gondii* infection employ the use of animal culture, cell culture, and histology. Sabin (1942) described the successful use of mice, guinea pigs and rabbits. However, although mouse inoculation (MI) is very sensitive, it is also very time consuming (Holliman 1988), taking up to six weeks to obtain a positive result. Cell culture can be carried out in monolayer cultures of human epithelial cells such as H.Ep-2 cells (Bickford and Burnstein 1966), and also in MRC5 and Vero cells (Chimpitazi *et al.* 1987). Parasite has been successfully isolated from cases of congenital toxoplasmosis using amniotic fluid (Derouin *et al.* 1988), and also from immunocompromised patients (Hofflin and Remington 1985, Calico *et al.* 1991, Tirard *et al.* 1991, Derouin *et al.* 1987 and Dannemann *et al.* 1992). Histology is less time consuming than culture, but it is not so sensitive. Conventional staining techniques include haematoxylin-eosin (HE) and Wright-giemsa. Luft *et al.* (1984) found that these stains missed organisms in 50% of cases. The most effective immunohistochemical techniques include peroxidase-anti peroxidase (Conley and Jenkins 1981) and alkaline phosphatase which can detect free tachyzoites, tissue cysts and phagocytosed organisms.

The most common methods used in diagnosis of toxoplasmosis involve serology, and several commercial kits are available. The dye test (DT), developed by Sabin and Feldman in 1948 is still used by reference laboratories today. The test, based on a complement mediated neutralising antigen / antibody reaction requires live organisms and fresh complement, making it complicated and expensive. There are also risks associated with the use of live parasite (Fleck 1989b). Methods such as the complement fixation test (CFT), enzyme-linked immunosorbent assay (ELISA) and direct agglutination test (DAT) are also used to detect IgG and IgM (McCabe and Remington 1983, Candolfi *et al.* 1987). Whole organisms are used in the DT, DAT and immunosorbent agglutination to detect antibodies to membrane antigens. These are most reactive early in infection. Other tests including CFT, ELISA, latex agglutination test (LAT) and indirect haemagglutination (IHA) use disrupted parasite. In the LAT, which is the most widely available commercial test, antigen is fixed to latex beads. Agglutination occurs when specific antibody is added and this test has good correlation with the DT (Johnson J. 1992), although false positive results have been recorded (Holliman *et al.* 1989). Such tests do not generally detect infection as early as those using whole parasite, but they remain reactive for a longer period of time. This means that these tests would be of less value in prenatal screening regimes. The IHA test has shown cases of false negative results, and the end point of titration is often difficult to read (Fleck 1989a). The ELISA system was seen as the test of choice by Hughes (1985) as it requires little technical expertise, can be easily assessed, and is suitable for automation. Johnson J. (1992) reported satisfactory sensitivity when compared to DT, but variability in specificity and quantification capabilities. However the ELISA did not detect primary toxoplasmosis as early as the LAT, and is therefore better suited to the detection of previous exposure to infection (Sutehall and Wreghitt 1989). Joss *et al.* (1989a) found IgG ELISA's to be sensitive, detecting 86-91% of infected samples. Biotin-labelled *T. gondii* antigen was used with avidin labelled with peroxidase in an IgM capture ELISA (BAM-ELISA) by Joss *et al.* 1989b). This was found to be 100% sensitive and therefore useful for a screening test. IgA was found more frequently than IgM in cases of congenital toxoplasmosis by Decoster *et al.* (1991), and so they recommended the use of this test in combination with others to give a rapid diagnosis.

It has been suggested that strain variation may account for antibody diversity in patients (Weiss *et al.* 1988a) and studies involving the screening of new recombinant antigens for use in diagnostic serology have been carried out (Suzuki and Remington 1990, Parker *et al.* 1991 and van Gelder *et al.* 1993). Some of these have been aimed at the detection of different stages of infection, for example to detect only the tachyzoite (Decoster *et al.* 1988, Suzuki *et al.* 1988a, 1988b and 1990). These involved the use of acute-stage specific antigens of *T. gondii* for serodiagnosis of acute infection. Results show that the system is sensitive and specific with 92% of sera obtained within two months of onset giving positive results, compared with 9% giving positive results after five months (Suzuki *et al.* 1990).

Hassl and Aspock (1990) used immunofluorescent testing (IFAT) and western blotting to monitor IgG and IgM response in AIDS patients. Although sera recognised several bands, a pattern of antigens diagnostic of acute infection was not found. Potasman *et al.* (1986) reported that serum antibodies to certain antigens appeared to follow acute infection. They found an early antibody response to a 4 kDa antigen and 35 kDa antigen which may be of value in dot blot analysis. As many of the antigens are stage specific (Kasper 1989), they may be of value in the differentiation of stages of infection.

Serological testing is useful in most patients. However it is of limited value in confirming congenital cases of toxoplasmosis, and in immunocompromised patients who have a limited ability to produce antibody. This is discussed further in section 1.9. There are very few tests available for the detection of *T. gondii* antigen, and none is commercially available (Wilson and McAuley 1991). Woodson and Smith (1990) reported that P30 could not be detected in tissue cysts in samples of brain and that there is strain variation in cyst and bradyzoite antigens. This implies that P30 would be a suitable antigen to use in a detection system for tachyzoites and therefore an active or acute infection.

The first antigen ELISA for detection of *T. gondii* was developed by van Knapen and Panggabean (1977), and since then several other systems have been published. These will be discussed in 1.9.1. Their problem is that they appear to lack sensitivity (Wilson and

McAuley 1991) and as the duration of parasite in acute infection is brief, the diagnostic value remains to be proven (Barker and Holliman 1992).

Dot-immunobinding analysis of antigens was capable of detecting 40-130 pg of antigen in mock samples, and also detected parasite in four out of six CSF samples from congenitally infected infants (Brooks *et al.* 1985). Others have used western blot analysis to characterise further antigens (Couvreur *et al.* 1988). Western blotting is a useful tool for determining antigens at different stages of infection, but is not easily transferred to a diagnostic setting (Wilson and McAuley 1991).

Advances in DNA technology have resulted in new methods of diagnosis such as the detection of parasite nucleic acid by dot blot hybridisation (Angel *et al.* 1992, Blanco *et al.* 1992). These systems could not detect less than 1000 parasites in samples of infected tissue, blood, cerebrospinal fluid (CSF) and amniotic fluid. A probe developed from a genomic *T. gondii* library was found to be insensitive, with an ability to detect 3 ng of parasite (Savva 1989).

1.8.3 Treatment

A wide variety of drugs have been assessed for the ability to treat human toxoplasmosis. Spiramycin, a macrolide antibiotic is used to treat a primary infection during pregnancy because it can readily cross the placenta (Desmonts and Couvreur 1974). However its efficacy and safety have not been fully evaluated (Jeannel *et al.* 1990).

Currently the drug of choice is pyrimethamine, a substituted phenylpyrimidine compound, which can be used on its own at rates between 0.5 and 1 $\mu g\ ml^{-1}$. However it is even more effective at lower levels when used in combination with sulphonamides, with which it has a synergistic effect (Harris *et al.* 1988). Pyrimethamine readily binds to protein, it is lipophylic, and it has a half life of 35 to 175 hours before being cleared by hepatic metabolism. It operates by the inhibition of tetrahydrofolic acid formation by interfering with dihydrofolate reductase (Weiss *et al.* 1988b). These drugs are useful in treating congenital toxoplasmosis because they also readily cross the placenta (Hall

1992). Clindamycin and its analogues have also been used in combination with pyrimethamine and found to be effective treatments of toxoplasma encephalitis (Luft *et al.* 1993), although Jacobson *et al.* (1992) had to discontinue part of a trial assessing clindamycin treatment due to 40% of patients suffering side effects including diarrhoea and rash.

If diagnosed early, patients usually respond rapidly to pyrimethamine / sulphonamide therapy. Using sulfadiazine (1 - 2 g 4x day) and pyrimethamine (25 - 50 ng 1x day), Wanke *et al.* (1987) found clinical improvement in 11 out of 13 HIV positive patients with toxoplasma encephalitis, and a reduction in brain lesions in 8 out of 10 patients. Improvement was noted over the first 2 to 4 weeks, but recrudescence occurred 14 to 42 days after stopping therapy. This reveals the problems which arise when treatment is suspended in the immunocompromised host. By blocking folic acid metabolism, only the tachyzoite is affected, and so the bradyzoites inside tissue cysts remain untreated and are able to reactivate when treatment is stopped or reduced. Treatment was stopped in 6 out of 13 patients due to toxic effects including neutropenia, fever and rash (Wanke *et al.* 1987). Leport *et al.* (1989) found that toxicity was less of a problem in a study using pyrimethamine with clindamycin where only one out of 11 patients was affected. As pyrimethamine is toxic to bone marrow, supplemental therapy with folic acid is necessary. Other treatments such as rehydration and dose reduction may also be necessary (Weiss *et al.* 1988b, Luft and Remington 1992).

As problems with relapse in immunocompromised patients are significant, maintenance therapy is routinely given after clinical improvement. This consists of continued lower daily doses of sulphadiazine and pyrimethamine, or pyrimethamine on its own. Pedrol *et al.* (1990) reported an incidence of 50% relapse in patients not receiving maintenance therapy, however in the study carried out by Wanke *et al.* (1987) relapse occurred in only 16.6% of patients receiving maintenance therapy. The use of intermittent maintenance therapy is therefore thought to be desirable, even if some relapse does occur. Similar results were obtained by Porter and Sande (1992) with 22% relapsing with, and 60% of patients relapsing without maintenance therapy.

The high levels of toxicity associated with these drugs highlights the need for less toxic agents in the treatment of toxoplasmosis. The need for drugs affecting the bradyzoites in tissue cysts was recognised by Huskinson-Mark *et al.* (1991) and Luft and Remington (1992). This would allow only one course of therapy and end the need for life long treatment and so reduce the problems associated with toxicity. They tested azithromycin, aprinocid-N-oxide and the hydroxynaphthoquinone 566C80 for effects on the bradyzoite *in vitro* with some success. The definitive action of 566C80 is unknown but it appears to target the mitochondrial respiratory chain, blocking electron transport. From the subsequent success of these drugs in *in-vivo* animal models it was concluded that further study in patients was warranted. The success of 566C80 in the treatment of *Pneumocystis carinii* in rats and mice as well as *T. gondii* infection would be of value as both infections are frequently encountered in patients with AIDS (Mills 1986).

Vaccination has had a limited role in the prevention of *T. gondii* infection, and so far it has been limited to veterinary use (1.7.1). For vaccines to be used successfully in the human population several problems need to be overcome. Firstly there is a need to find a vaccine which does not consist of live parasite, for example in the form of a protective recombinant antigen. This could then be manufactured in a suitable vector. *Salmonella* was suggested as a vector by Bulow and Boothroyd (1991) but the public distrust associated with this organism may prevent its use for human vaccine production. Secondly, there is a need to identify a susceptible population, as it would not be possible to control the massive animal reservoir of infection. As a result, humans not vaccinated would still be at risk.

1.9 DIAGNOSIS IN HIGH RISK GROUPS

Accurate and rapid diagnosis of infection in pregnancy (Holliman *et al.* 1991a) and in AIDS patients is essential for patient management and improved prognosis (Blanco *et al.* 1992, Luft *et al.* 1983b). Antibody testing is of little value in cases of reactivation in immunocompromised patients if there is an absence of specific immunoglobulin (Mills 1986, Wanke *et al.* 1987), and *T. gondii* antigenaemia and parasitaemia are rarely detected. Dannemann *et al.* (1991) followed 33 patients for between one and 14

months, and failed to detect parasite using MI and antigen ELISA, even in samples from two patients within 45 days of developing toxoplasma encephalitis. The importance of the detection of antigenaemia and parasitaemia remains unclear (Vendrell *et al.* 1993). As a result of this, diagnosis in these patients is often made on the basis of clinical findings, including the use of computer axial tomography (CAT) and magnetic resonance imaging (MRI). These scans can reveal brain lesions (Navia *et al.* 1986), but are far from conclusive as similar effects can be due to HIV itself or to infection by other organisms such as Cytomegalovirus (CMV), Herpes simplex, *Mycobacterium spp, Cryptococcus spp, Candida albicans* and *Aspergillis fumigatis* (Luft and Remington 1988, Wanke *et al.* 1987). Therefore an important part of a diagnosis is to exclude other pathogens. Confirmation by pathological examination of a brain biopsy is an invasive technique, and is not considered acceptable in most situations (Barker and Holliman 1992). However it is considered to be necessary by Wanke *et al.* (1987) who rejected non-invasive techniques as not being sufficiently sensitive, particularly for patients who do not respond to therapy (Holliman *et al.* 1991b)

Management and prevention are therefore important in the control of the illness. IgG status is of value in locating patients at risk from reactivation of toxoplasmosis and HIV positive patients with negative *T. gondii* serology should be given advice on avoiding primary infection. Patients who have been exposed to the parasite should be monitored for any neurological changes, especially in cases of advanced AIDS, (Luft and Remington 1992). As identification of those at risk minimises delay in diagnosis and therefore permits early therapy, routine screening using MRI and CAT scans and blood samples should be implemented (Grant *et al.* 1990), particularly if abnormal clinical signs are encountered in the CNS. Lumbar puncture and screening for IgG and IgM in the CSF collected should also be carried out in conjunction with testing for other organisms (Mills 1986). Although CSF may appear normal, Potasman *et al.* (1988) recognised the diagnostic value of testing for intrathecal antibody. If a diagnosis cannot be made, an aggressive regime is warranted, including brain biopsy and empiric therapy (Wong *et al.* 1984). MRI and CAT scans are both useful in the assessment of response to therapy,

when evidence of improvement should be seen within three weeks if toxoplasmosis is the cause (Luft and Remington 1992).

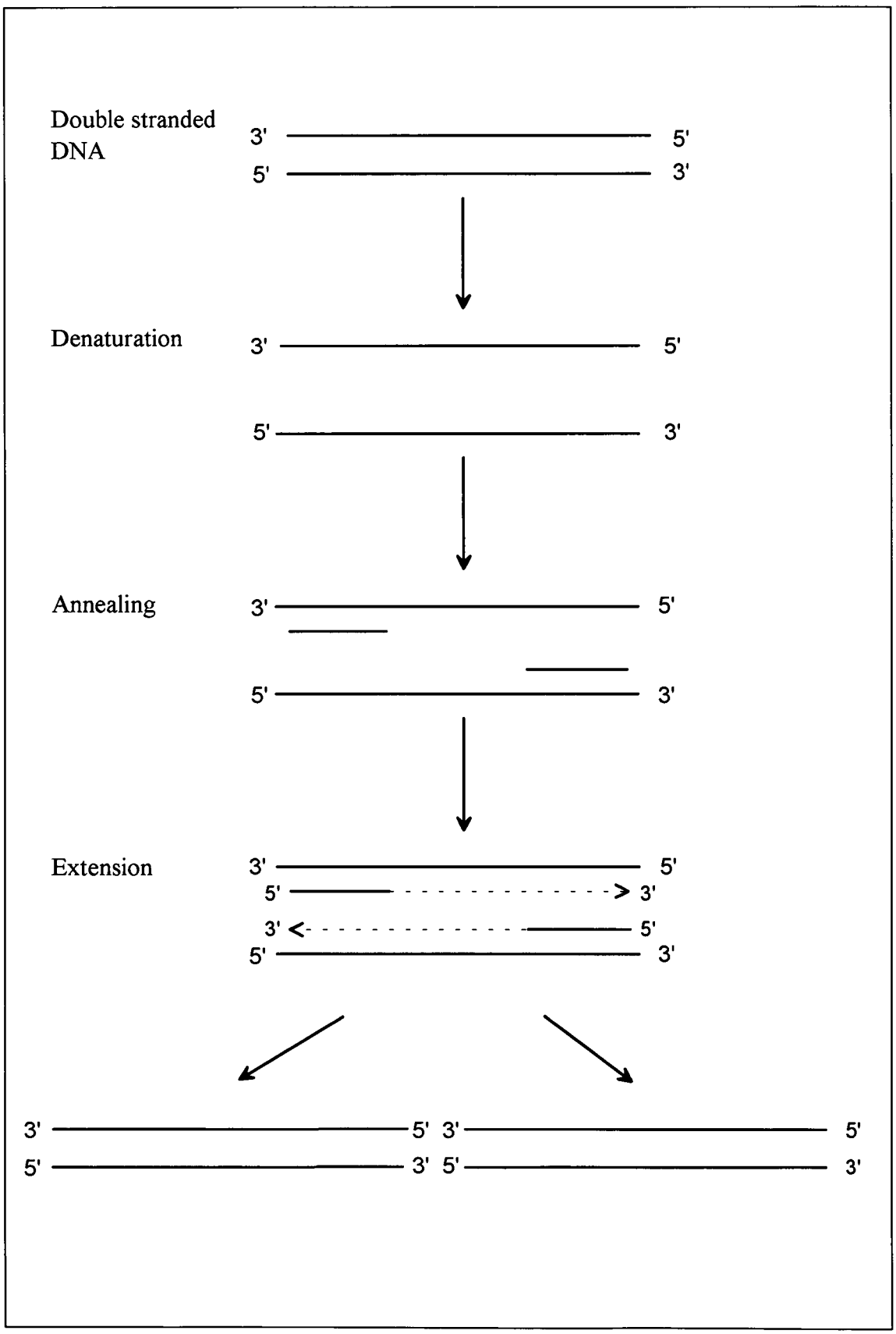
It is apparent that more sensitive methods of direct testing involving non-invasive procedures are necessary. The failure to detect parasitaemia and antigenaemia may be due either to the limitations of the tests used, or because the parasite is circulating intermittently (Dannemann *et al.* 1991), or transiently (Barker and Holliman 1992). The development of a more sensitive antigen detection ELISA and tests using DNA technology have been suggested for use with samples such as blood and urine. These would be much easier to obtain than biopsy specimens, cause less trauma to the patient, and could be collected sequentially, especially from patients being treated.

The antigen ELISA has been developed over a number of years for the detection of *T. gondii* in samples from a wide variety of animals. Success rates varied greatly and so the standardisation of such a test would be necessary for its use in successfully diagnosing cases of toxoplasmosis where others have failed (van Knapen and Panggabean 1977, Araujo and Remington 1980, Lindenschmidt 1985).

The polymerase chain reaction (PCR) was developed by Mullis in 1983, but not published until 1985 by Saiki *et al.* due to patent applications. This powerful method has been applied to the detection of small amounts of specific nucleic acid from a wide variety of organisms. These include CMV (Shibata *et al.* 1988), *Pneumocystis carinii* (Lipschik *et al.* 1992) and Papillomavirus (Morris *et al.* 1988). It is also capable of detecting genetic disorders and is used as a research tool and in forensic science (Kitchin 1990).

Amplification of specific nucleic acid occurs through a series of cycles of heating and cooling carried out in a programmable heating block. Double stranded DNA is first denatured by heating to a high temperature. Both strands can serve as a template for synthesis of DNA, therefore specific primers are added which flank the area to be amplified. When the temperature is reduced these primers anneal to the complementary

Figure 1.3 The Polymerase chain reaction.



part of the appropriate strand and are then used as the starting point for the creation of new strands of DNA. This requires the presence of building blocks of deoxynucleoside triphosphates (dNTP's) and DNA polymerase, which catalyses the reaction. The polymerase used is a thermostable *Taq* DNA polymerase is obtained from *Thermus aquaticus*, which maintains its efficiency over many cycles of temperature variation. After 25-30 cycles the amount of target DNA will have increased exponentially and can be detected by dot-blotting or electrophoresis (see Figure 1.3) (Kitchin 1990).

1.9.1 Antigen ELISA

Van Knapen and Panggabean (1977) carried out a study to investigate the use of an ELISA system to detect antigen. This involved 1 116 sera from individuals suspected of having toxoplasmosis, from which antigen was detected in 64 (5.7%). As circulating antigen (cag) was detected in so few patients suspected of having toxoplasmosis, it was suggested that antigen circulates for only a short period. Thus they reported that cag could only be found in the short active phase of primary infection and during reactivation and concluded that when carried out with antibody testing, antigen ELISA could provide valuable information on the present stage of infection. If antibody levels were high (IFAT>1024), and cag was detected, then they suggested that the cause of illness was a primary infection, and if antibody was low (IFAT<512, CF and IgM negative) with detectable cag, then the infection was considered to be in the latent stage. They also emphasised the advantage of obtaining a positive result with the antigen ELISA on the basis of one sample, in contrast to the requirement for paired sera when testing for antibody.

A similar antigen detection ELISA was also used successfully by Araujo and Remington (1980) to test serum samples from 22 patients diagnosed as having acute toxoplasmosis. In this case purified $F(ab)_2$ fragment of IgG raised from *Toxoplasma* infected rabbits was used to coat the plate resulting in a detection rate of 63.6% (14 out of 22 patients). Araujo and Remington (1980) concluded that parasitaemia may only be present intermittently as 36.4% of cases of diagnosed acute toxoplasmosis failed to produce a positive result using this technique.

By 1985 interest in a system to detect *T. gondii* antigen had increased with the rising number of AIDS patients suffering from toxoplasmosis. As antibody testing is of limited value in immunocompromised patients an alternative system of diagnosis was required in these individuals.

Lindenschmidt (1985) studied 51 patients with lymphadenopathy and found that 42 were *T. gondii* IgM positive. Out of these 42 patients, 30% were antigen positive by ELISA. This system used 3 M NaSCN to disassociate antigen / antibody immune complexes which were recognised as a problem in detection systems (Neurath *et al.* 1982). High levels of circulating antibody may bind antigen in serum thus making it unavailable for detection in the ELISA system. Using the system of van Knapen and Panggabean (1977) only 14.3% of samples were positive. Therefore Lindenschmidt (1985) concluded that, contrary to earlier finding, immune complexes may cause problems resulting in reduced sensitivity.

Further work by van Knapen, Panggabean and Leusden (1985) used ELISA to investigate circulating immune complexes (CIC) in mice which had been experimentally infected with *T. gondii*. CIC were found by day 3 post infection (p.i.) with the RH strain. Using the non-virulent T626, strain which is probably more relevant to human infection, cag was detected for a very short period. This supported the hypothesis that free antigens only circulate for a limited period before CIC are formed. CIC were found from day 13 p.i. up to day 35. By day 75 the only evidence of past infection was antibody. Therefore in the mouse model the presence of CIC appears to parallel the acute and sub-acute stages of infection.

The study by van Knapen *et al.* (1985) also compared serum from patients with suspected toxoplasmosis, and serum from patients with confirmed toxoplasmosis, and from a control group of blood donors. CIC was detected in 12.5% of patients with suspected toxoplasmosis, 37.5% of those with confirmed toxoplasmosis, and also in several of the control group. The number detected in the control group may have been due to a high level of infection in the population of The Netherlands where this study

was carried out. The final part of this study involved following a case infection resulting from a laboratory accident. In this case the level of cag was too low to be considered positive by ELISA. The individual had high levels of IgG and IgM, so the low amount of cag was explained by the formation of CIC, which was detected at initial infection and also at a later stage of infection. Van Knapen *et al.* (1985) noted that CIC represents the presence of active parasitaemia or acute toxoplasmosis and concluded that the detection of CIC may be indicative of active infection. However, as they are often detected in cases of asymptomatic infection, the test is of limited diagnostic value.

A more refined ELISA using avidin-biotin (ABELISA) was developed by Ise *et al.* (1985). This was assessed as a possible method of detecting cag by using it to monitor experimentally infected rabbits. This system did not involve a dissociation step and could detect 4 ng ml^{-1} which compared well with the 30-50 ng ml^{-1} detection rate obtained by Araujo and Remington (1980). Improved detection was due to the high degree of affinity of avidin for biotin which is 10^6 times that of the affinity of antibody for antigen (Subba Reo *et al.* 1983). The system detected the RH strain 3 days post infection, after which it rose sharply until day 8-9 when all rabbits were dead. Using the Beverley strain, antibody levels increased with time, but all samples were antigen negative until the end of the study when all animals were still alive. Therefore the system missed what was concluded to be low levels of infection of the non-virulent strain.

Transplant patients showing signs of *T. gondii* reactivation were studied by Candolfi *et al.* (1987). Antigen was detected in 14 out of 115 sera tested from 19 patients (12.2%). They found antigen in sera from patients with acute toxoplasmosis, whereas sera from chronically infected patients were negative, and they concluded that the presence of cag was associated with an active infection. Antigen was detected in the blood of 7 patients prior to the detection of symptoms, but antigenaemia appeared to be limited or at a very low level and they concluded that it was confined to patients suffering a reactivation of infection.

Samples of serum and CSF from HIV positive patients were tested for antigen by Hassl *et al.* (1988). They used the purified IgG $F(ab)_2$ fragment to coat the ELISA plate in a similar method to Araujo and Remington (1980) and detected cag in 16% of patients, most of whom had progressed to the advanced stage of AIDS (stage iv). Symptoms of toxoplasmosis were found in only 13 of these patients and only 2 had detectable IgM. No cag was found in CSF, even with a toxoplasma encephalitis was present. The significance of detectable antigen in patients with AIDS was questioned by Hassl *et al.* (1988), who disagree with Araujo and Remington (1980) and van Knapen and Panggabean (1977) in their description of antigenaemia as being indicative of acute infection.

Antigen was detected in the sera and urine from acutely infected mice using ELISA and western blot by Huskinson, Stepick-Biek and Remington (1989). All these studies have demonstrated several methods of using ELISA to detect antigen, including possible ways to increase sensitivity, but have not proven useful for the diagnosis of *Toxoplasma* encephalitis (Luft and Remington 1988). To this end it has been suggested that the use of monoclonal antibodies against secreted / excreted antigens may be useful for the detection of cag in human samples. An antigen ELISA was developed for use with sheep samples by Maley (1991), but had limited sensitivity and a level of 300 ng of antigen had to be present before a positive result was recorded. Part of the aim of the following investigation is to explore the possibility of increasing the sensitivity of the antigen ELISA for the detection of *T. gondii* in human samples.

1.9.2 Polymerase Chain Reaction

Several systems have been described which use the PCR for the specific amplification of *T. gondii* DNA to levels detectable by gel electrophoresis. PCR was first investigated by Burg *et al.* (1989) for use in the diagnosis of toxoplasmosis with the B1 gene (see 1.6) as the target. Quantitative Southern Blot analysis revealed that between 25 and 50 copies of this gene are present in the different strains of *T. gondii*, making it an ideal choice for amplification studies. Grover *et al.* (1990) found the gene to be present in all examined strains of the parasite (twenty one) demonstrating that it is highly conserved. Using purified *T. gondii* DNA, the system was found to be sensitive enough to detect a single

parasite after hybridisation with a labelled probe. The system was highly specific and did not amplify DNA from *Sarcocystis, Neospora, Plasmodium, Aspergillis, Candida, Cryptococcus* or *Absidia* spp.. However the system lost some sensitivity when used on mock clinical samples. In these samples, ten parasites could be detected in the presence of 10^4 leukocytes but it was concluded that the system needed further optimisation to increase sensitivity for clinical samples. Initial studies by Burg *et al.* (1989) used only one amplification of 25-60 cycles to give a 193 or 150 base pair (bp) product. The system has since been developed using nested amplifications (Lebech *et al.* 1992, Ho-Yen *et al.* 1992 and Verhofstede *et al.* 1993) to increase sensitivity.

Several studies have been carried out to investigate the possible use of the B1 PCR in the diagnosis of toxoplasmosis. These have involved the use of mock samples containing known amounts of DNA (Joss *et al.* 1993), and animal samples (Hitt and Filice 1992) The latter study compared B1 PCR to MI and cell culture. Mouse inoculation detected parasite in 62% of samples compared to 37% by B1 PCR and 25% by cell culture.

The B1 PCR has also been used to detect *T. gondii* from human samples. In 1990 Verhofstede *et al.* reported its use to diagnose congenital toxoplasmosis in an infant using a CSF sample collected 10 days after birth. This was not confirmed by serological testing until 15 months later. Grover *et al.* (1990) investigated amniotic fluid from 43 cases of acute maternal infection. B1 PCR was positive in eight out of ten samples, whereas *Toxoplasma* IgM was detected in three samples, and tissue culture was positive in four samples. They highlighted the need for better methods of sample preparation as cell debris was shown to inhibit PCR. In the two samples negative by B1 PCR, large numbers of red blood cells (RBC's) were present. They concluded that use of the PCR in cases of acute infection in pregnancy could reduce the number of unnecessary terminations presently carried out. The B1 PCR was used to detect toxoplasmosis in three congenitally infected children, and also in post mortem (PM) tissues by van de Ven *et al.* (1991). Microscopy, tissue culture and MI were also investigated. The PCR gave results compatible with MI, only failing to detect parasite in one sample positive by MI.

However, Weiss *et al.* (1992) found that the B1 PCR often failed to detect *T. gondii* in tissue samples which had been stored frozen.

Lebech *et al.* (1992) and Parmley *et al.* (1992b) studied CSF from 13 and 14 patients respectively using the B1 PCR. Lebech *et al.* (1992) detected parasite in two patients and Parmley *et al.* (1992b) detected *Toxoplasma* in four patients. No *Toxoplasma* DNA was detected in control samples.

Parasite was detected in blood samples from three out of seven cases of reactivated infection where serology proved inadequate (Ho-Yen *et al.* 1992). However infection was not detected in 13 patients with ongoing toxoplasma lymphadenopathies. PCR results were supported by MI, and this further suggests that only a transient parasitaemia is present in acute infection in healthy individuals.

The B1 PCR was used to detect *Toxoplasma* DNA in aqueous humour in three cases of ocular toxoplasmosis (Brezin *et al.* 1991). The B1 gene target has also been used by Groß *et al.* (1992) to amplify a larger fragment using different primers. Of 52 tissue and amniotic fluid samples collected from five patients ten samples were shown to contain *Toxoplasma* DNA by the B1 PCR. All but one of these was confirmed by MI.

The other DNA target which has attracted much attention is the gene coding for the P30 surface protein (Burg *et al.* 1988). This system was developed by Savva *et al.* (1990) using six different isolates from mouse culture to detect 0.5 pg of DNA after amplification, followed by gel electrophoresis and hybridisation studies. This was improved to detect 0.05 pg after a further amplification using nested primers. However, when mock samples were used, a smear of artefact bands often made the 522 bp product difficult to interpret when viewed on a gel. This was resolved by southern blotting and hybridisation. The system was also specific for *T. gondii* when tested against *Hammondia hammondii, Cryptosporidium parvum, Giardia lamblia* and *Eimeria vermiformis.*

The P30 PCR has been used to detect *T. gondii* in equine eyes, (Turner and Savva 1991) and in ovine tissue samples (Turner *et al.* 1991). Human samples have also been used successfully (Savva and Holliman 1990). These include brain biopsies from AIDS patients (Holliman *et al.* 1990b, Holliman *et al.* 1991b)

The P30 PCR was used to detect infection in cardiac transplant patients by Holliman *et al.* (1992). In a comparison between this technique and histology of cardiac biopsies from transplant patients, the PCR detected *Toxoplasma* DNA in eight samples whereas histology only detected *Toxoplasma* in two cases. Blood samples from AIDS patients were investigated by Dupouy-Camet *et al.* (1993) and in six out of seven proven cases of toxoplasmosis the P30 PCR gave a positive result, and 13 out of 14 samples from cases of presumptive toxoplasma encephalitis were also positive. Out of those followed up after treatment, several were still positive after 17 days of therapy. None of the 17 control cases were shown to be positive. They concluded that the P30 PCR was a suitable tool for the diagnosis of cerebral and disseminated toxoplasmosis and that it may also be useful for monitoring the effects of treatment. Johnson *et al.* (1993) reported that the P30 PCR was more sensitive than cell culture and MI in samples from pregnant women, foetuses, neonates, AIDS patients and transplant patients. The P30 PCR was also employed by Dupouy-Camet *et al.* (1990) using different primers to create a 282 bp fragment and this was used successfully to detect parasite in samples from cases of suspected congenital infection. However several confirmed cases were negative with the test. This was explained by the P30 target gene containing only one copy which could be missed in samples containing very small numbers of parasite.

Studies comparing different PCR methods have been limited. Both the B1 and P30 PCR were used to investigate ocular samples from two patients by Brezin *et al.* (1990). B1 PCR detected parasite, but P30 PCR did not. A larger study was carried out by Verhofstede *et al.* (1993) on 33 CSF samples including nine from confirmed cases of toxoplasma encephalitis. The B1 PCR was positive in six out of nine samples whereas the P30 PCR only detected two positive samples. They suggested that the higher copy number of the B1 gene may account for the greater sensitivity of this test.

Other PCR systems have also been attempted. Ribosomal DNA (rDNA) was used as a target for amplification by Cazenave *et al.* (1991), to produce an 88 bp fragment over 35 cycles, and Guay *et al.* (1993) amplified DNA from one parasite using rRNA as a target. A system for amplifying complimentary DNA (cDNA) was developed by Weiss *et al.* (1991), but this was found to be less sensitive than either the B1 or P30 PCR methods (Verhofstede *et al.* 1993).

1.10 AIMS

The aim of the following study was to develop a sensitive method of detecting *T. gondii* in the immunocompromised host. Such a method would be of great diagnostic value and ideally would utilise samples obtained using non-invasive procedures. Specific diagnosis would reduce the need for further investigations and would ensure the prompt initiation of appropriate treatment. Two PCR systems and an antigen ELISA were investigated for this purpose, and to monitor the effects of therapy. A successful system would ensure that treatment was specific and appropriate and so minimise the risk of toxicity due to high doses of therapeutic agents. Such a system would also be of benefit in the investigation of parasite / host interaction with particular emphasis on the mechanisms involved in recrudescence.

CHAPTER 2

Preliminary Investigations:
The use of the PCR and Antigen ELISA to detect *T. gondii* Infection

2.1 INTRODUCTION

This initial investigation involved the development of the ELISA for the detection of *T. gondii* antigen and the PCR to detect *T. gondii* DNA. In the latter, methods using the B1 and P30 genes were developed as reports indicated that both are useful in the detection of *T. gondii* DNA from purified parasite and in infected human and animal samples (Holliman *et al.* 1991b, Turner *et al.* 1991). However these studies lacked adequate controls, and neither had been assessed for a role in diagnosis. While the PCR technique is relatively expensive and requires specialised laboratory facilities, various ELISA systems are already in widespread use throughout the world for the diagnosis of a range of organisms. An antigen ELISA would therefore be of value for the diagnosis of toxoplasmosis as it would be easy to implement in most diagnostic laboratories.

2.2 MATERIALS AND METHODS FOR THE PCR

2.2.1 Frequently used solutions

Unless otherwise stated reagents were obtained from Sigma. Suppliers are listed in appendix I.

PBS:	137 mM NaCl, 26.8 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4.
TE :	10 mM Tris (pH 7.5), 0.1 mM EDTA, unless stated otherwise.
Phenol :	Equilibrated with 1 M Tris-HCl (pH 8), then with 0.1 M Tris HCl (pH 8). B-hydroxyquinoline was added to 0.1% (w/v) and stored at -20°C.
Chloroform:	Chloroform was mixed with isoamyl alcohol at a ratio of 24:1 (v/v) and stored in a dark bottle at room temperature.
Phenol / Chloroform:	Phenol and chloroform were mixed 1:1 and stored at 4°C.
Proteinase K:	Stock was made up at 20 mg ml^{-1}, aliquoted and stored at -20°C.
RNase A:	Stock was made up at 10 mg ml^{-1}, heated to 100°C for 15 minutes, cooled to room temperature and stored at -20°C.
20x SSC:	3 M NaCl, 0.3 M sodium citrate, stock solution adjusted to pH7 with concentrated NaOH.
5x Loening "E":	900 mM Tris, 750 mM Na H_2PO_4, 25 mM EDTA, pH7.6-7.8.

2.2.2 Parasite Preparation

Parasite was passaged in Swiss White mice every three days by intraperitoneal inoculation with 10^6 RH strain tachyzoites. Peritoneal exudate was removed and the parasite cleaned by washing with PBS followed by Hanks Balanced Salt Solution (Gibco) and then centrifuged at 500 *g* for 5 minutes. Parasites were counted using a haemocytometer and preparations containing a 98% tachyzoite suspension were used. (Work carried out by J. Wastling).

2.2.3 DNA Extraction from *T. gondii*

DNA was extracted from 10^9 *T. gondii* tachyzoites for use in developing the PCR and for future positive control material. Parasite was lysed in 50 mM Tris (pH 8.0), 50 mM EDTA, 1% SDS, containing 100 μg ml^{-1} proteinase K. After incubation at 50°C for three hours, the nucleic acid was extracted with an equal volume of phenol / chloroform followed by an equal volume of chloroform. DNA was then precipitated with 2 volumes of ethanol (100%) and 0.1 volume of 3 M sodium acetate at -20°C for 1 hour, followed by centrifugation at 10 000 *g*. The pellet was washed in 70% ethanol and then resuspended in TE. RNA was removed by incubation with 20 μg ml^{-1} RNase for 30 minutes at 37°C and the DNA was re-extracted and precipitated as above.

2.2.4 DNA Extraction from CMV

(Supplied by S. Burns, City Hospital). This method was adapted from those of Brytting *et al* (1991) and Sandin *et al.* (1991). Aliquots of virus were frozen and thawed three times, digested using pronase (100 μg ml^{-1}), followed by phenol /chloroform and chloroform extraction. DNA was precipitated with ethanol (see 2.2.3) and the pellet was resuspended in 100 μl TE.

2.2.5 DNA Extraction from *Candida albicans*

(Supplied by S. Burns, City Hospital). This method was adapted from Monod *et al.* (1990). Cells were harvested and washed with 50 mM EDTA (pH 8), followed by centrifugation at 600 *g*. The pellet was resuspended in 150 μl of 50 mM EDTA and 80 μl lyticase solution (2 ng lyticase in 10 mM Na PO_4 (pH 8), 50% (v/v) glycerol) and

incubated at 37°C for 20 minutes. Proteinase K (1 mg ml^{-1}) was added in NDS buffer (0.5 M EDTA, 10 mM Tris pH 7.5, 1% (w/v) sodium laurylsarcocinate) and the digest was incubated at 50°C for 24 hours. DNA was extracted using phenol / chloroform, followed by chloroform extraction and precipitated with ethanol as before. The pellet was redisolved in TE and treated with RNase (20 $\mu g\ ml^{-1}$) and re-extracted (see 2.2.3). The final pellet was redisolved in 100 µl TE.

2.2.6 DNA Extraction from *Sarcocystis* spp. and *Pneumocystis carinii*

(*Sarcocystis* supplied by N. Lally, Moredun Research Institute; *Pneumocystis carinii* supplied by S. Burns, City Hospital). This was carried out as for *T. gondii* extraction.

2.2.7 DNA Extraction from *Aspergillis fumigatus*

(Supplied by S. Burns, City Hospital). This was carried out using the method of Denning *et al.* (1990). Cells were centrifuged at 1200 *g* and washed with 10 ml of 0.6 M Mg SO_4. The pellet was resuspended in 3 ml buffer (1.2 M Mg SO_4 buffered with 10 mM K PO_4 to pH 5.8). This buffer was also used to prepare stock solutions of cellulase and Novozyme 234 at concentrations of 20 mg ml^{-1}. Equal proportions of each enzyme, 0.5 ml g^{-1} wet weight of pellet were added, and cell wall digestion was carried out by shaking at 33°C for 60 - 90 minutes. The suspension was overlaid with separation buffer A (0.6 M sorbitol, 100 mM Tris (pH 7)) and centrifuged at 1 500 *g* for 1.5 minutes. The protoplast layer was then removed from the interface and resuspended in 5 ml separation buffer B (1.2 M sorbitol, 10 mM Tris, 50 mM EDTA (pH 7.5)), and centrifuged at 800 *g* for 10 minutes. Finally, the supernatant was removed and the pellet was washed twice and then resuspended in 5 ml buffer B.

DNA was then extracted by adding 0.5 ml lysis buffer (2% (w/v) SDS, 50 mM EDTA pH 7.8). After mixing and incubating at 70°C for 30 minutes, the preparation was centrifuged at 8 000 *g* for 5 minutes. The pellet was then digested with 100 µl proteinase K (100 $\mu g\ ml^{-1}$ in distilled water) at 50°C for 1 hour. This was followed by phenol / chloroform extraction and ethanol precipitation (see 2.2.3).

2.2.8 Quantification of DNA

The concentration of DNA preparations was assessed by taking readings of OD_{260} using a spectrophotometer (OD_{260} equal to 1 corresponds to 50 μg ml^{-1} double stranded DNA). Readings were also taken at OD_{280}. DNA preparations free from protein possessed an OD_{260} : OD_{280} ratio approaching 2.0.

2.2.9 Basic Reaction Conditions

The PCR was carried out in a Techni PHC3 (Scotlab) thermal cycler over 30 cycles in 50 μl volumes which were overlaid with 50 μl of mineral oil. Both PCR systems employed the use of nested primers. Primers were obtained from Oswell DNA Services, dNTP's from Pharmacia, and *Taq* DNA Polymerase from Boehringer Mannheim. Samples were initially denatured for 10 minutes at 95°C before the first amplification cycle to ensure that any DNA present was fully denatured.

2.2.10 The P30 PCR

The method of Savva *et al.* (1990) was used to amplify the gene coding for the *T. gondii* P30 surface protein. To optimise this system fully, titrations were carried out using varying concentrations of $MgCl_2$, KCl_2, dNTP's and primers. The final conditions used over the rest of the study were in fact very similar to those of Savva, and were as follows: The reaction mixture consisted of 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM MgCl, 0.1% (w/v) gelatin, 0.2 mM dNTP's, 0.2 μM each primer and 2.5 units of *Taq* DNA polymerase. The primers used in the system were as outlined in Figures 2.1 and 2.2. The first amplification was carried out using primers DS29 and DS30 to produce a fragment of 914 bp, followed by a nested amplification using primers DS38 and DS39. The fragment produced from the second amplification was 522 bp long. The cycle regime was as follows: denaturation at 95°C for 1 minute, annealing at 60°C for 1 minute, and extension at 72°C for 3 minutes.

2.2.11 The B1 PCR

The method of Burg *et al.* (1989) was modified to amplify the multi-copy B1 gene. Titration of the components of the system revealed critical concentrations of MgCl and

Figure 2.1 Primers for the P30 PCR

DS29. TTG CCG CGC CCA CAC TGA TG

DS38. CGA CAG CCG CGG TCA TTC TC

DS39. GCA ACC AGT CAG CGT CGT CC

DS30. CGC GAC ACA AGC TGC GAT AG

Figure 2.2 Positions of the primers on the P30 gene

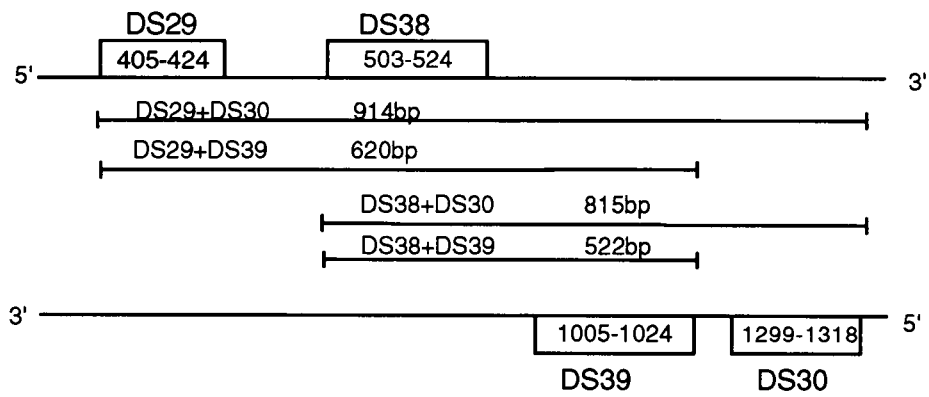


Figure 2.3 Primers for the B1 PCR

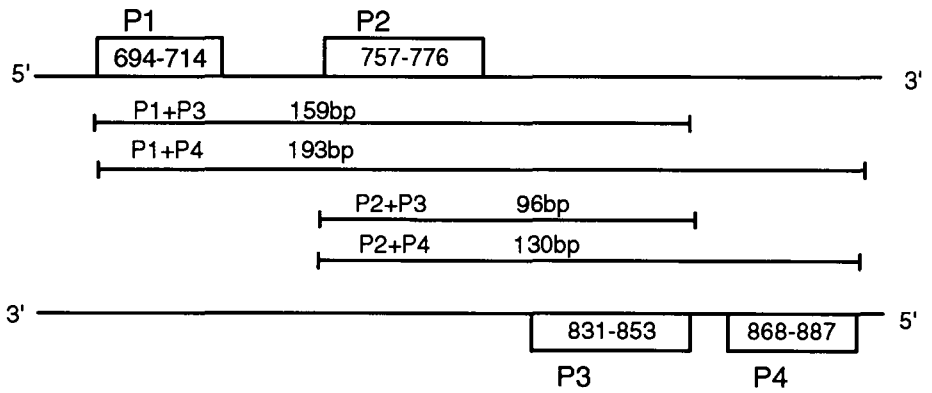
P1. GGA ACT GCA TCC GTT CAT GAG

P2. TGC ATA GGT TGC AGT CAC TC

P3. GGC GAC CAA TCT GCG AAT ACA CC

P4. TCT TTA AAG CGT TCG TGG

Figure 2.4 Positions of the primers on the B1 gene



KCl were necessary to fully optimise the reaction. The final conditions of the system were therefore quite different from those used by Burg, and were as follows: 10 mM Tris (pH 8.3), 2.5 mM MgCl, 40 mM KCl, 0.01% gelatin, 0.2 mM dNTP's, 0.2 μM of each primer and 2.5 units of *Taq* DNA polymerase. Various primer combinations were used (see Figures 2.3 and 2.4) with P2 being used without the T7 site and containing only 20 bases. In the initial reaction P1 and P4 amplified a 193 bp product. When this was followed by a nested amplification using P2 and P3, the product obtained was only 96 bp. The cycle regime consisted of denaturation at 95°C for 1 minute, annealing at 50°C for 1 minute and extension at 72°C for 3 minutes.

2.2.12 Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) of DNA was based on the method of Sambrook *et al.* (1989). Acrylamide stock (29.2% (w/v) acrylamide, 0.8% (w/v) bisacrylamide) diluted with Loening "E" buffer was used to make 7.5% polyacrylamide gels. The reaction was catalysed by 0.1% (v/v) N,N,N',N',-tetramethylethylenediamine (TEMED), and 0.1% (w/v) ammonium persulphate (APS) (Biorad), and the gels were poured immediately after their addition. Gels were cast using the "mini protean" gel system (Biorad) and run at 150 volts constant setting.

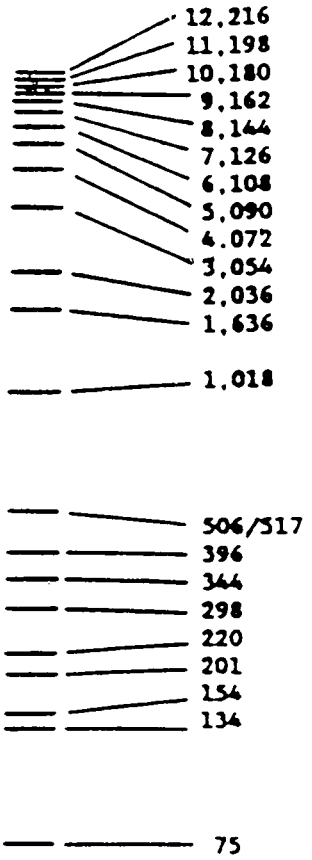
2.2.13 Silver Staining of Polyacrylamide Gels

DNA in polyacrylamide gels was stained using a modification of the method of Herring *et al.* (1982). Gentle agitation was used for all the steps of the staining procedure. The gel was first fixed in 100 ml of 10% (v/v) ethanol and 0.5% (v/v) ethanoic acid for 10 minutes, followed by staining in 11.2 mM $AgNO_3$ for 10 minutes. After washing twice in distilled water the bands were developed by the addition of 0.75 M NaOH containing 0.25% (v/v) formaldehyde for a further 10 minutes. The reaction was stopped when the bands became clearly visible by removing the solution and adding 75 mM Na_2CO_3.

2.2.14 Agarose Electrophoresis of DNA

Agarose "sub-cell" gel tanks were supplied by Pharmacia. Nucleic acid grade "ultrapure" agarose (BRL) or molecular grade (Sigma) was used at concentrations of 1 - 1.2%

Figure 2.5 KB Ladder



(w/v) in Loening "E" buffer. Ethidium bromide (Et Br) was added to molten agarose at a final concentration of 0.2 μg ml^{-1}. DNA loading buffer (0.1% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 75 mM EDTA, 25% sucrose) was added 1 : 9 (v/v) to samples prior to loading, and electrophoresis was carried out at 2 - 5 volts cm^{-1} in Loening "E" buffer until the required resolution was achieved. Co-electrophoresis of a 1 Kb ladder (Gibco - BRL) permitted the estimation of band size (Figure 2.5).

2.2.15 Southern Blotting of DNA to Nylon Membranes

DNA was transferred from agarose gels to nylon membranes using a modification of the method of Smith and Summers (1980). The gel was denatured in 0.5 M Na OH, 1.5 M NaCl for 30 minutes, and then neutralised in 1 M ammonium acetate for 1 hour. "Hybond N" nylon membrane (Amersham) was then placed on top of the gel followed by 3 sheets of Whatman 3MM chromatography paper which had been soaked in neutralising solution. A further 3 sheets of dry chromatography paper were added, and finally a 3 cm stack of paper towels and a 1 kg weight were placed on top of the membrane. Transfer was carried out over 3 - 18 hours. The filter was then washed in 2 x SSC, dried, and the DNA fixed to the membrane by ultra-violet (UV) irradiation (0.4 J cm^{-1}).

2.2.16 Preparation of Digoxigenin Labelled DNA Probes

PCR products were labelled with digoxigenin (DIG) using the "DIG DNA labelling and detection kit" (Boehringer Mannheim). A fragment of 96 bp (using primers P2 and P3) was used for the B1 system, and a fragment of 522 bp (using primers DS38 and DS39) for the P30 system. The DNA was denatured by boiling for 10 minutes, then incubated with 1 μl random hexanucleotide mix, 1μl dNTP labelling mix and 1 unit of Klenow enzyme for 2 - 16 hours at 37°C. This labelling reaction results in the random incorporation of DIG - 11 - dUTP every 20 - 25 nucleotides in the newly synthesised DNA (Feinberg and Volgenstein 1983).

Alternatively, PCR was used to prepare large quantities of DIG - labelled probes. PCR reactions were carried out as described in 2.2.10 and 2.2.11, with DIG- dUTP incorporated into the 2 mM dNTP x10 stock at a ratio of 35 : 65 (DIG - dUTP : dNTP).

2.2.17 Hybridisation of DIG Labelled Probes

Hybridisations were carried out as recommended by the manufacturers (Boehringer Mannheim). Filters were prehybridised by incubating in hybridisation solution (5 x SSC, 5% (w/v) "blocking reagent" , 0.1% (w/v) N- lauroylsarcosine Na- salt, 0.02% (w/v) SDS, 50% (v/v) formamide) for 1 hour at 42°C. Freshly denatured probe was then added (2 - 5 µl) and hybridisation was carried out for 2 - 16 hours at 42°C. Filters were washed twice in 2 x SSC, 0.1% (w/v) SDS for 5 minutes, then twice in 0.1 x SSC, 0.1% (w/v) SDS for 15 minutes at 68°C.

2.2.18 Detection of Bound DIG Labelled Probes

All steps were carried out at room temperature. Filters were washed briefly in Buffer 1 (100 mM Tris, 150 mM NaCl) and then blocked in Buffer 2 (Buffer 1 with 0.5% (w/v) "blocking reagent") for 30 minutes. After washing, the filters were incubated in 10-20 ml of Buffer 1 containing 1:5000 (v/v) dilution of anti-DIG alkaline-phosphatase conjugate for 30 minutes. Any unbound conjugate was removed by washing twice in Buffer 1 for 15 minutes. The filters were then equilibrated in Buffer 3 (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$ pH 9.5) and a colour solution was added which consisted of 45 µl "NBT" solution (75 mg ml^{-1} nitroblue tetrazolium salt in dimethylformamide), and 35 µl "x-phosphate" (50 mg ml^{-1} in 5- bromo-4-chloro-3-indolylphosphate, toludinium salt in dimethylformamide) in 20 ml Buffer 3. The filters were incubated in the dark for 1 - 24 hours until the colour developed. The reaction was stopped by washing briefly in TE and the filters were air dried.

2.3 MATERIALS AND METHODS FOR THE ANTIGEN ELISA

2.3.1 Antigen Preparation

Soluble *T. gondii* antigen was obtained by freezing and thawing a clean tachyzoite suspension (2.2.2) three times. Particulate matter was then removed by centrifugation at 10 000 *g* for 30 minutes and the soluble fraction stored at -20°C until required.

2.3.2 Raising Antibody

Three New Zealand White rabbits were inoculated intramuscularly with 4 x 10^5 S48 tachyzoites from mouse peritoneal exudates in Freund's incomplete adjuvant. Blood was collected from each every two weeks and the serum separated and tested by IFAT until the IgG antibody titre reached 1: 64 000. IgG in the serum was extracted by passing pooled serum through a Staphylococcal protein A column (Goding 1976). The column was washed with PBS and the IgG fraction was eluted out with 0.58% glacial acetic acid containing 0.15 M sodium chloride. Ultra-violet absorption was used to identify the IgG peak and the corresponding fractions were collected and dialysed overnight at 4°C with PBS, and stored at -20°C.

2.3.3 Commercial Antibody

Monoclonal anti- *T. gondii* antibody was supplied by Quadratech.

2.3.4 Conjugate

Horseradish Peroxidase (HRP) conjugated to the IgG molecule had been prepared earlier (Maley 1991) using the method of Wilson and Nakane (1978). Briefly, Grade V1 HRP (Sigma) was added to sodium meta periodate and the resulting aldehyde was dialysed against 1 mM sodium acetate buffer (pH 4.4) overnight at 4°C. Sodium carbonate (pH 9.5) was then added to raise the pH of the solution to pH 9. Rabbit anti- *Toxoplasma* serum was added (2 ml fractions), and the mixture was incubated at room temperature for two hours. Sodium borohydrate (0.4 mg ml^{-1}) was then added and incubated at 4°C for two hours. Absorbance readings were taken at 280 nm and 403 nm and the best fractions were pooled and bovine serum albumin (BSA) was added to 10 mg ml^{-1}. The conjugate was then divided into 0.5 ml aliquots and stored at -20°C.

2.3.5 Assay Procedure

Dynatech M129B plates were coated with 150 µl of rabbit anti-IgG antibody diluted at 1:4000 in 0.1 M carbonate / bicarbonate buffer (pH 9.6). Commercial monoclonal antibody (2.3.3) was used at 1: 500. Plates were incubated overnight at 4°C and then washed three times in PBS containing 0.05% Tween 20 (PBST). A series of control

antigen dilutions (doubling dilutions from 1 : 200 to 1: 25 600) were included, and 150 µl of serum or control was added to the appropriate well and the plates were incubated at 37°C for two hours. After three washes with PBST, rabbit anti- *T. gondii* IgG was added at 1 : 5 000 in PBST containing 1% BSA and incubated at 37°C for a further two hours. The plates were again washed and 150 µl of O-Phenylenediamine (OPD) substrate was added for 30 minutes. The substrate consisted of 40 mg OPD in 100 ml phosphate citrate buffer (0.02 M citric acid, 0.05 M sodium hydrogen phosphate pH 5) plus 40 µl hydrogen peroxide. The reaction was stopped using 50 µl 2.5 M sulphuric acid and the plates were read using a 492 nm filter on a Titretek ELISA reader.

2.3.6 Samples

As well as using a range of diluted antigen controls and dilutions containing from 1-1000 parasites (approximately, see 2.4), ELISA was carried out on samples from sheep experimentally infected with 10^5 *Toxoplasma* tachyzoites and from patients showing clinical signs of toxoplasmosis.

2.3.7 Acid Dissociation of Samples

150 µl of serum was added to 150 µl of 1.5 M glycine (pH 1.85). After vortexing, the mixture was incubated at 37°C for 1 hour and then neutralized with 150 µl of 1.5 M Tris (pH9). The ELISA was then carried out using 150 µl of this sample using the procedure in 2.3.5 differing only in sample incubation time which was increased from 1 hour to overnight at 37°C.

2.4 COMPARISON OF THE PCR AND ELISA

Both antigen ELISA and PCR were used against a panel of control samples infected with a known amount of *T. gondii* parasites (200 µl PBS containing 1000, 500, 100, 50, 10, 5, or 1 parasites). Samples were used neat for ELISA using the protocol in 2.3.5. DNA was phenol / chloroform extracted and ethanol precipitated (2.2.3) for use in B1 and P30 PCR (2.2.10 and 2.2.11).

2.5 RESULTS

2.5.1 Sensitivity of the PCR

When P30 PCR was used against a titration of *T. gondii* DNA controls, the system was found to detect down to 0.5 pg on a 1% agarose gel (Figure 2.6). This sensitivity was improved by southern blotting to detect 0.05 pg of DNA (Figure 2.7). The B1 PCR appeared to be slightly more sensitive than P30 as 0.05 pg could often be detected as a faint band on 1% agarose gels (Figure 2.8). This was not further improved by Southern blotting, although bands were more intense (Figure 2.9). However the result was often difficult to interpret because of the close proximity of the 96 bp product to the primer haze which was often present when the sample was run on a gel (2.10). Bands were therefore better differentiated by PAGE (Figure 2.11).

2.5.2 Specificity of the PCR

DNA from CMV, *Candida albicans*, *Sarcocystis spp.*, *Pneumocystis carinii* and *Aspergillus fumigatus* did not result in amplification products by B1 or P30 PCR.

2.5.3 Optimisation of the PCR

As was described in 2.2.10 and 2.2.11 it was possible to optimise both PCR systems to detect low levels of parasite. Because the nested primers (P2 and P3) produced a fragment of only 96 bp in the B1 PCR which was difficult to differentiate from the primer haze on agarose, the nested amplification was carried out using P1 and P3 to give a product of 158 bp which was easier to differentiate. An additional problem was the number of artefact bands which were present when products were run on a gel although these bands were not present after Southern blotting. This problem was overcome by including a dilution step between the initial amplification and the nested amplification. A dilution factor of 1:20 in water was sufficient to reduce the amount of background bands to a negligible level.

Figure 2.6 P30 PCR products by agarose gel electrophoresis (Et. Br) using decreasing amounts of *T. gondii* DNA template. Products were detected down to 0.5 pg.

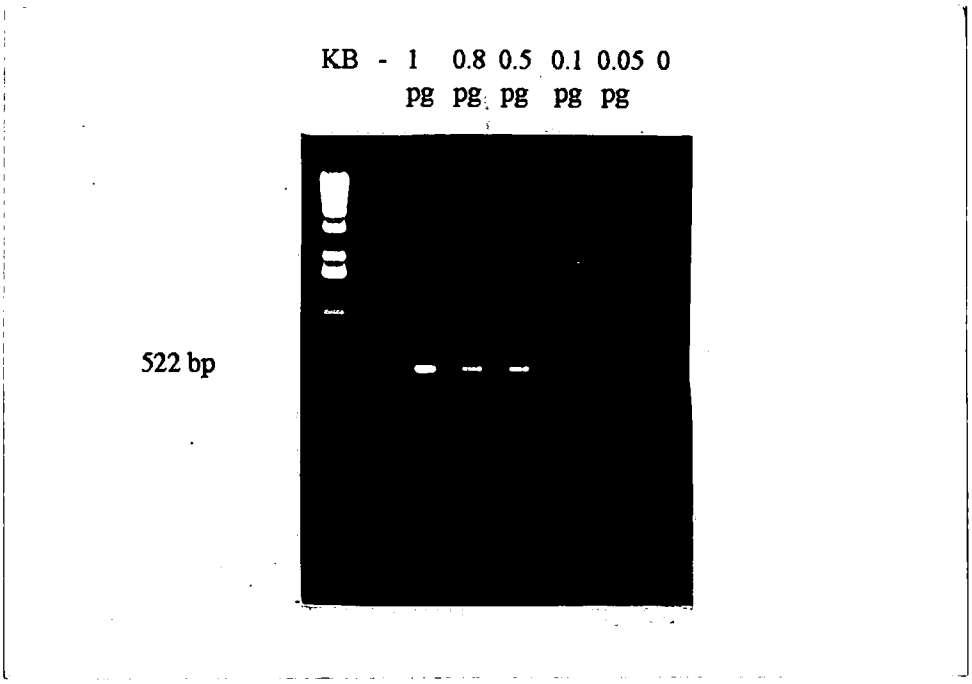


Figure 2.7 P30 PCR products by Southern blot from agarose gel (Figure 2.6) showing an increase in sensitivity to detect 0.05 pg

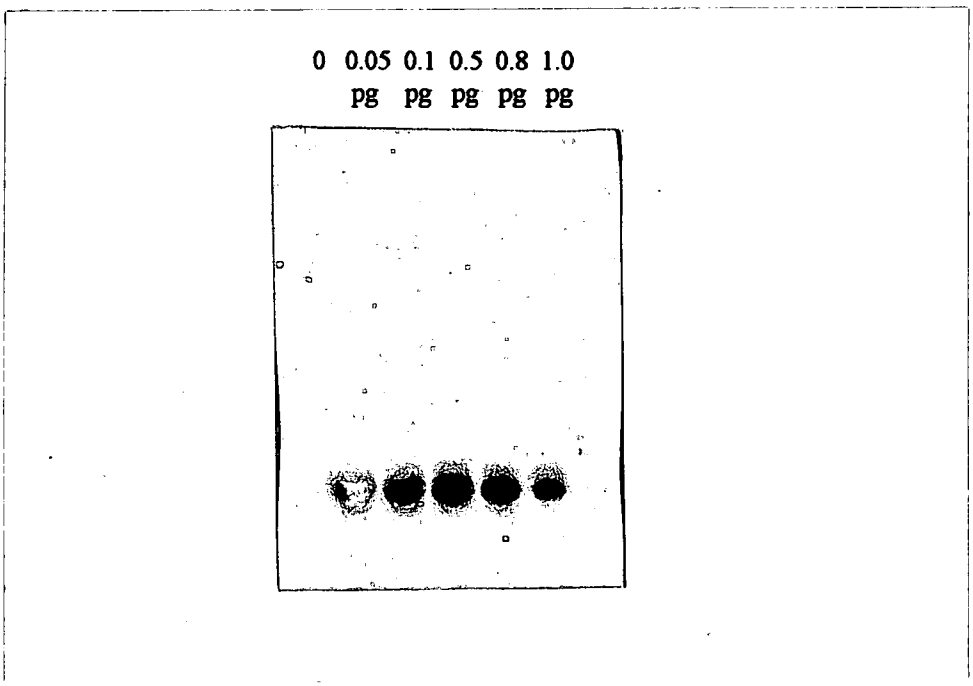


Figure 2.8 B1 PCR products by agarose gel electrophoresis (Et. Br) using decreasing amounts of *T. gondii* DNA template.

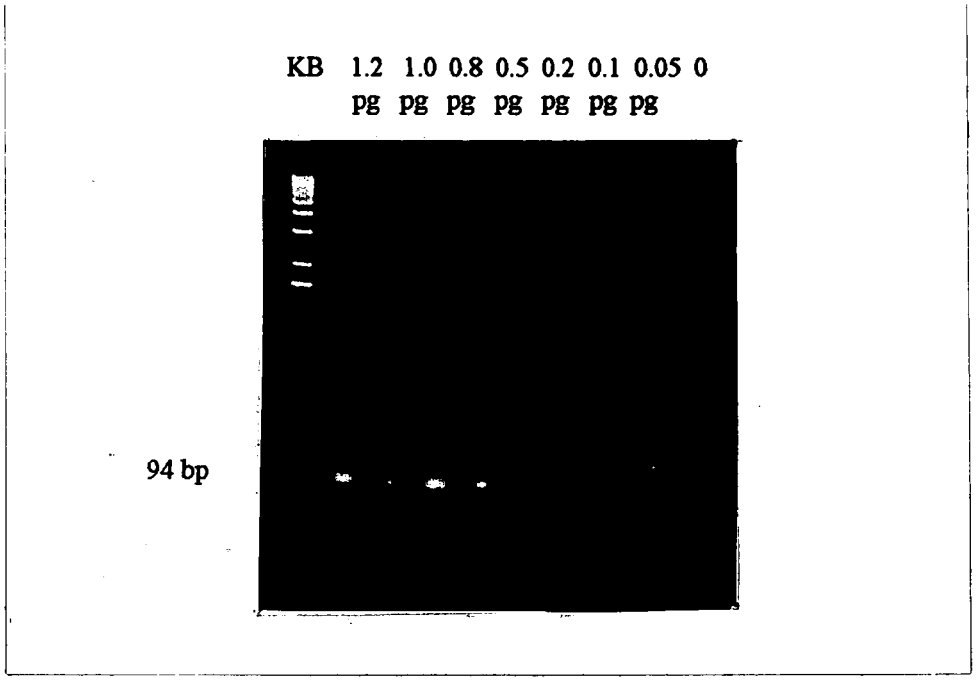


Figure 2.9 B1 PCR products by Southern blot from agarose gel (Figure 2.8).

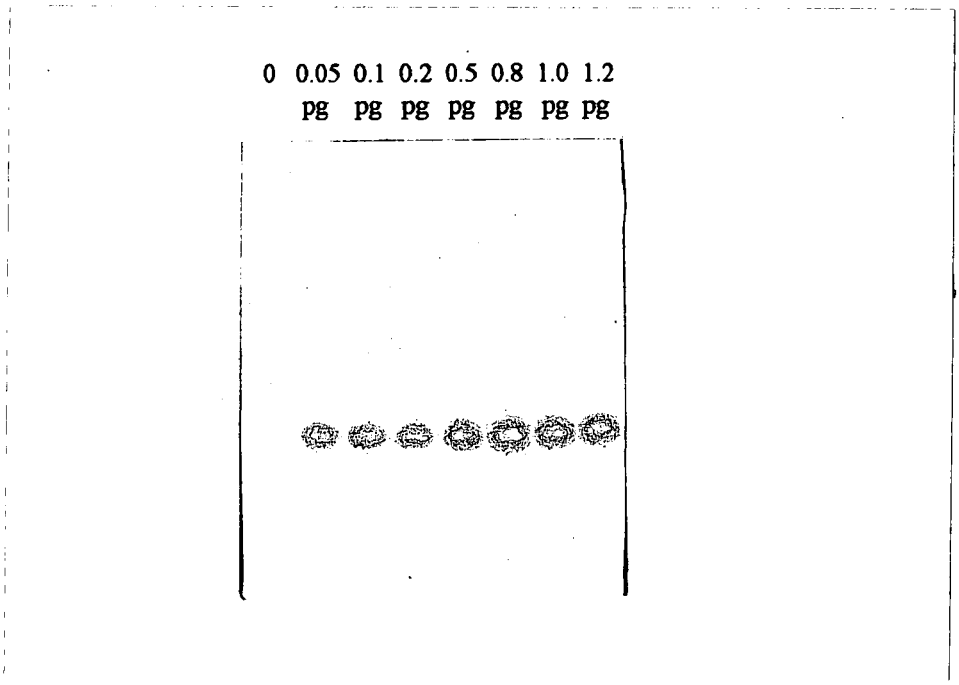


Figure 2.10 B1 PCR products by agarose gel electrophoresis using different primer combinations.

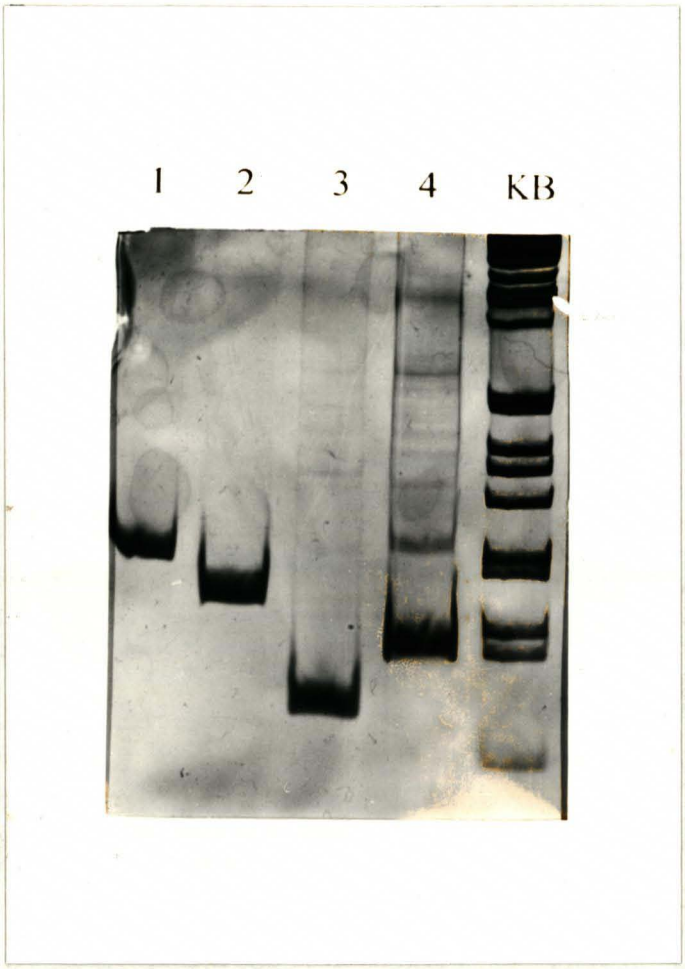
Lane 1: P1 + P4 (193 bp)

Lane 2: P1 + P3 (159 bp)

Lane 3: P2 + P3 (96 bp)

Lane 4: P2 + P4 (130 bp)

Figure 2.11 B1 PCR products by PAGE using different primer combinations



Lane 1: P1 + P4 (193 bp)

Lane 2: P1 + P3 (159 bp)

Lane 3: P2 + P3 (96 bp)

Lane 4: P2 + P4 (130 bp)

It was possible to reduce the running time of the reactions by decreasing the time for each stage of the cycles and still obtain the sensitivity to detect 0.05 pg DNA. The final cycle regime used for the B1 PCR was 95°C for 20 seconds, 50°C for 20 seconds and 72°C for 40 seconds. This reduced the total running time from 3 hours to 1 hour 45 minutes for each amplification. There was also a reduction in time for the P30 PCR from 3 hours to 1 hour 55 minutes for each amplification. The final regime was 95°C for 40 seconds, 60°C for 40 seconds and 72°C for 1 minute.

2.5.4 Antigen ELISA

The ELISA using polyclonal antibody did produce a good standard curve of absorbance against dilution using antigen controls (Figure 2.12). However when the test was used on 10 samples from clinically infected sheep, none of the samples could be considered positive as all absorbance readings were below that of the standard curve. Similar negative results were obtained with 20 samples from patients with clinical symptoms of toxoplasmosis, although samples showed slightly raised levels. Acid dissociation of antigen / antibody complexes did increase the absorbance readings of sera, but did not detect any positive results with the samples used in this study (Figure 2.13).

Commercially produced monoclonal IgG produced a similar standard curve with antigen controls. However results were more promising as serum samples from two patients with clinical toxoplasma encephalitis were within this curve and could be considered positive, although these were at the lower end of the range (Figure 2.14).

2.5.5 Comparison of the PCR and ELISA

The PCR successfully amplified DNA from *T. gondii* in control samples down to 0.05 pg DNA, which is approximately equal to one parasite (Savva *et al.* 1990). The B1 PCR detected parasite at this level in 2 out of 3 cases on 1% agarose and by Southern blotting. P30 detected parasite at this level in 1 out of 3 cases by Southern blotting, although the band could not be seen on 1% agarose.

Figure 2.12 Antigen ELISA. Standard curve (best fit) of absorbance against dilution using antigen controls.

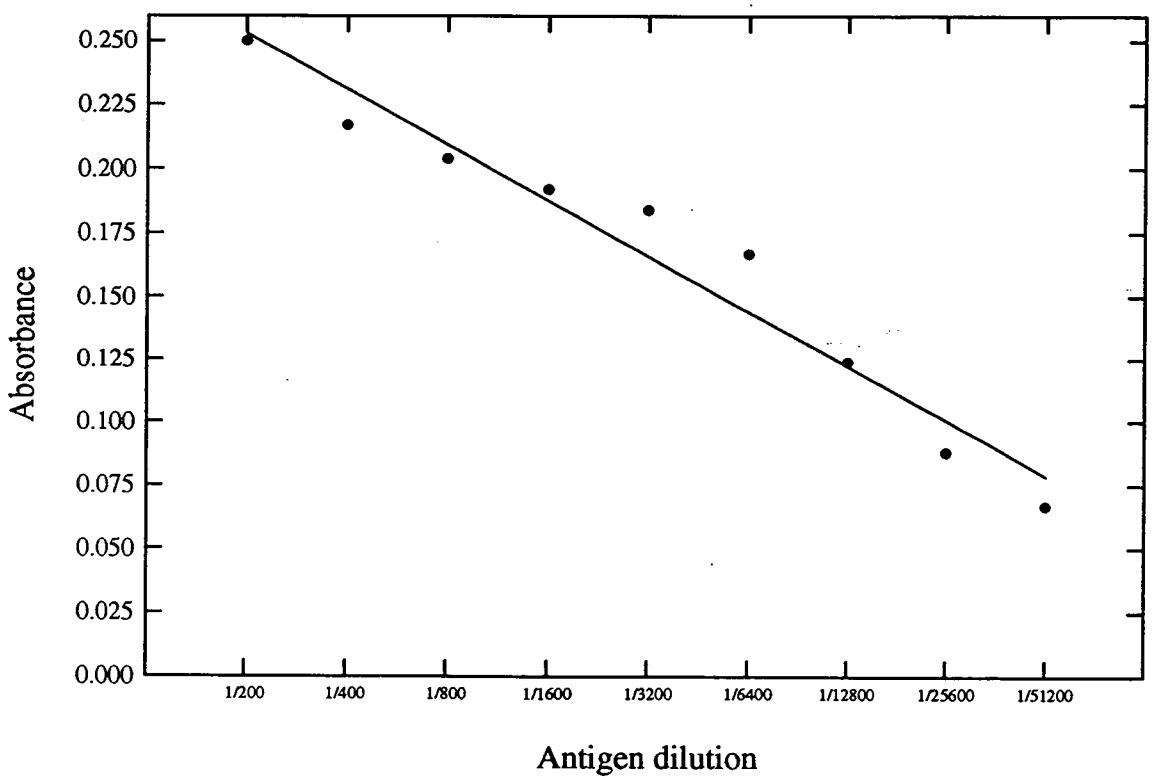


Figure 2 .13 Antigen ELISA. Standard curve (best fit) of absorbance against dilution using antigen controls after acid dissociation of antigen / antibody complexes.

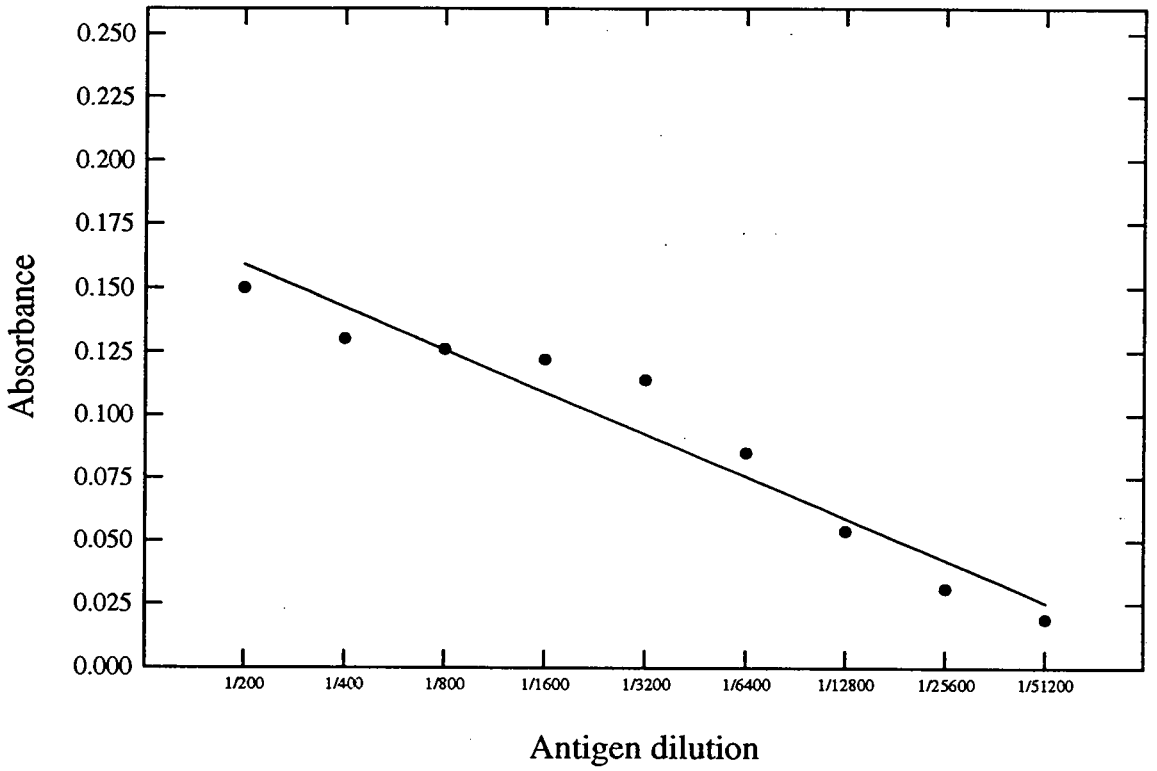
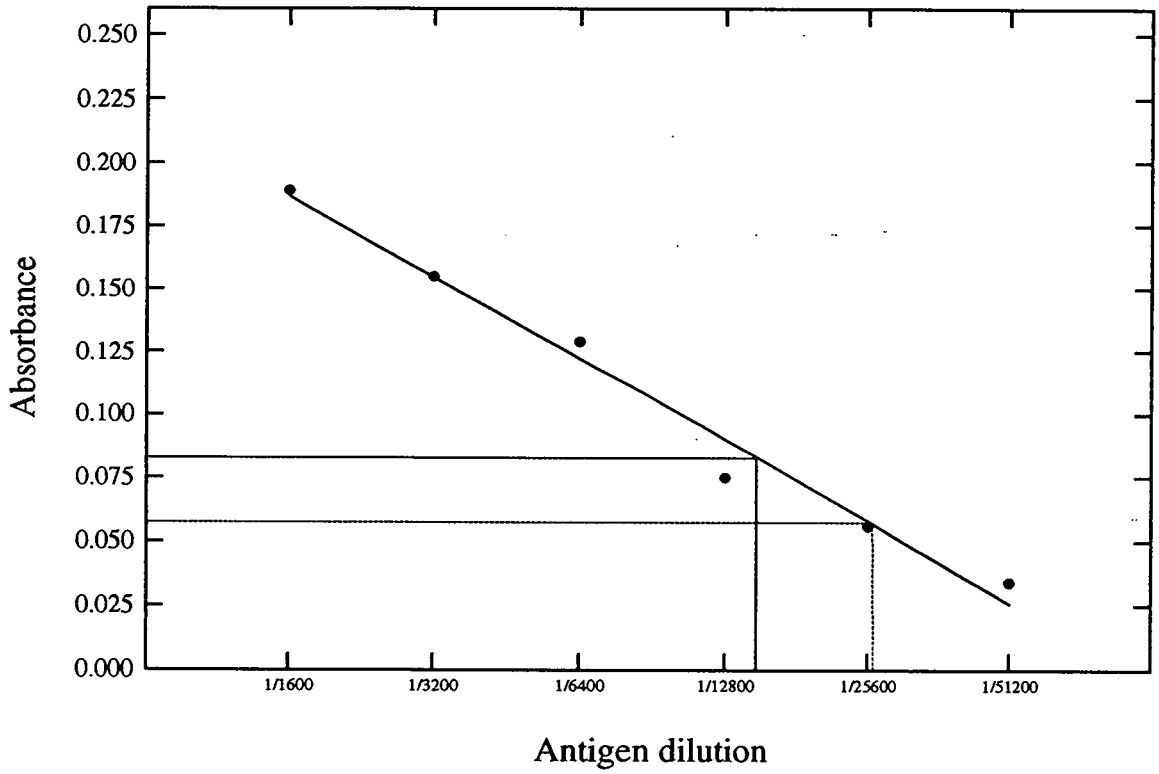


Figure 2.14 Antigen ELISA. Standard curve of absorbance against dilution with antigen controls using commercially produced monoclonal IgG. The results from the two patient sera which were positive are shown.



ELISA detected 500 parasites when monoclonal antibody was used to coat the plates, and only 1000 parasites when using polyclonal antibody. It did not detect parasite at lower levels (100, 50, 10, 5 or 1).

2.6 DISCUSSION

The antigen ELISA proved to be disappointing in its inability to detect *T. gondii* infection in both control samples and clinical samples, although the use of monoclonal antibody did improve performance to a level where infection was detected in two out of 20 clinical samples. The low sensitivity of the ELISA has also been reported by others using polyclonal sera or fractions of polyclonal sera. For example, van Knapen and Panggabean (1977) reported a detection rate as low as 5.7% in sera from individuals suspected of having acute toxoplasmosis. Antigenaemia was detected by Candolfi *et al.* (1987) in only 7 out of 22 patients who later seroconverted or showed clinical signs of toxoplasmosis. Araujo and Remington (1980) did demonstrate cag in 65% of patients with recently acquired toxoplasmosis by using affinity purified $F(ab)_2$ fragment to coat the plate. This may improve the sensitivity of the system, but it is unlikely to significantly increase performance. When this system was used by Hassl *et al.* (1988) antigen was detected in only 16% of AIDS patients and so they questioned the significance of cag in this population.

There may also be problems with interference by the presence of antigen-antibody complexes in the ELISA system (Hassl *et al.* 1988). Van Knapen *et al.* (1985) used an ELISA system to detect specific *T. gondii* complexes and obtained a positivity rate of 12.5% in individuals suspected of having toxoplasmosis. Lindenschmidt (1985) included an extra step to dissociate antigen-antibody complexes with 3 M NaSCN. This technique showed 30% of acute phase sera to be positive, compared to only 5% when the method of van Knapen *et al.* (1985) was used. Acid dissociation of these complexes did not improve the rate of positive results in this study.

The PCR performed well in the control specimens tested in this study. Both the B1 and P30 systems were sensitive, with the ability to detect down to the equivalent of only one

parasite. Although this degree of sensitivity was not possible on every occasion, this was likely to be due to sampling variation. Parasite numbers were recorded and then diluted to the various testing levels. At such low levels it would be possible to have no parasite, or one or two parasites in the small sample tested by the PCR. Both systems were also specific when tested against other organisms.

The sensitivity of the PCR was substantially better than that of the ELISA for the detection of *T. gondii* in the control samples. Although some factors of the ELISA, such as the use of monoclonal antibody may be improved to increase the sensitivity of the system, the ability to locate and amplify specific pieces of *T. gondii* DNA make the PCR the most effective method for use as a diagnostic tool. It was therefore decided to discontinue work on the ELISA in order to concentrate on the PCR.

The samples tested so far have been "spiked" buffer controls but it is necessary to carry out tests using clinical samples. Several studies have successfully used the B1 and P30 PCR to detect parasite in certain clinical samples (for example Weiss *et al.* 1991, van de Ven *et al.* 1991, Holliman *et al.* 1990b). However these have lacked controls in using only samples from patients with suspected toxoplasmosis. The sensitivity of either system has not been previously been demonstrated using controlled clinical samples. The next stage of this study therefore attempted to address this point.

CHAPTER 3

A Comparison of the B1 and the P30 Polymerase Chain Reaction

3.1 INTRODUCTION

Both the B1 and the P30 PCR were shown to amplify purified DNA from *T. gondii* (chapter 2). These systems were also successful when used by Turner *et al.* (1991), Burg *et al.* (1989) and Groß *et al.* (1992) in studies using limited samples. However none of these investigations included clinical samples from infected subjects studied over a significant time course, or included confirmation using other systems such as MI. In order to assess the suitability of the B1 and the P30 PCR for the detection of *T. gondii* in clinical samples, it was necessary to apply these techniques in controlled experimental conditions.

This study involved the comparison of the B1 and the P30 PCR by following the course of infection in sheep experimentally infected with *T. gondii*. The aim of the study was to establish whether one of the PCR methods was more suitable for use with clinical samples, and to compare the performance of this methodology against MI. Mouse inoculation is currently regarded as the most sensitive and accurate diagnostic test as it is believed that only one parasite is necessary to cause illness in an inoculated animal. It is therefore the 'gold standard' to compare against PCR.

As the B1 system targets a multi copy gene, it was hypothesised that this would be more sensitive than P30, which amplifies a single copy gene. Initial studies (chapter 2) revealed the B1 PCR to be more sensitive than the P30 PCR in the detection of purified DNA from a single parasite when viewed on an agarose gel, but both displayed equal sensitivity using Southern blot analysis.

In the second part of this study samples of blood, lymph and tissue were investigated for their suitability for the detection of *T. gondii*. Haemoglobin in blood is known to inhibit the PCR (van de Ven *et al.* 1991, Weiss *et al.* 1991, Joss *et al.* 1993), therefore three preparation methods were investigated to identify an appropriate system for amplification.

3.2 MATERIALS AND METHODS

3.2.1 Parasite Preparation

S48 tachyzoites were cultured as described in 2.2.2. Preparations containing 98% tachyzoites were used to inoculate sheep.

3.2.2 Samples

Scottish Blackface ewes (n=9) which were seronegative for *T. gondii* by IgG ELISA (Buxton *et al.* 1988) were inoculated with approximately 10^5 S48 tachyzoites by subcutaneous injection. Efferent lymph was collected from six animals following surgical cannulation of the pre-femoral lymph duct (Buxton *et al.* 1994 in press, see Appendix II) for MI, and for the B1 and P30 PCR. Peripheral blood samples were collected from the remaining three non-cannulated sheep for PCR only. Samples were taken prior to infection and then every 24 hours for 15 days post infection (p.i.). Tissue samples of brain, spleen, lymph nodes, diaphragm, liver and kidney were obtained at post mortem examination from one animal in which the cannula failed 12 days p.i..

3.2.3 Sample Preparation

Lymph samples were diluted 1:10 with PBS to prevent clotting and centrifuged at 800 *g* for 10 minutes. Contaminating red blood cells (RBC's) were removed using osmotic shock by resuspending the cell pellet in 10 mM Tris ammonium chloride (pH 7.2), followed by 3 washes in PBS. The pellet was then resuspended in 50 µl of K buffer (100 mM Tris pH 8.3; 500 mM potassium chloride; 15 mM magnesium chloride; 0.5% Tween 20) containing 100 µg ml^{-1} proteinase K. After incubating at 55°C for one hour the enzyme was inactivated by boiling for 10 minutes after which the samples were stored at -20°C until ready for use.

Blood samples were prepared using three methods:

1) Collection of the buffy coat from heparinised blood by centrifugation at 800 *g* over lymphoprep (Nycomed, Oslo). The Remaining RBC's were removed using 10 mM Tris ammonium chloride (pH 7.2) followed by centrifugation at 13 000 *g* for 30 seconds. The pellet was then resuspended in K buffer containing proteinase K and treated as above.

2) Dilution of 50-100 µl of blood in 10 mM Tris ammonium chloride (pH 7.2) to lyse RBC's, followed by centrifugation at 13 000 *g* for 30 seconds. The pellet was resuspended and washed a further two times before being resuspended in K buffer containing proteinase K and treated as above.
3) Dilution of 50-100 µl of blood in 0.5 ml of water and then boiling for 20 minutes.

Tissue samples, frozen prior to treatment, were finely dissected and washed in PBS. RBC's were removed as before and the tissues digested overnight at 37°C in 100 µl of 50 mM Tris (pH 8.5), 1 mM EDTA, 0.5% Tween 20 containing 200 µg ml^{-1} proteinase K. The enzyme was then inactivated, and the samples stored as before.

3.2.4 The PCR

This was carried out as described in 2.2.9 - 2.2.11.

3.2.5 Mouse Inoculation

Three female Swiss White mice were each inoculated with 250 µl of lymph by i.p. injection for each sample tested. The animals were inspected daily for signs of a febrile response that may indicate acute toxoplasmosis. Animals which appeared ill were culled immediately and a sample of peritoneal exudate removed and inspected for tachyzoites by microscopic examination. Mice which survived were culled at 8 weeks post inoculation and the presence of *T. gondii* antibodies detected by IgG ELISA (Buxton *et al.* 1988). (Work carried out by J. Wastling).

3.2.6 Statistical Analysis

A generalised linear model with a binomial error term (McCullagh and Nelder, 1983) was used to analyse data for parasite detection in lymph by PCR and MI. This was programmed in the Genstat 5 programming language (Payne *et al.* 1988). Parasite detection in blood by the B1 and P30 PCR was compared by a two-tailed *t* test.

3.3 RESULTS

3.3.1 Parasite Detection in Efferent Lymph

Table 3.1 shows MI and PCR results obtained from lymph from two cannulated animals which were typical of the group. It can be seen that samples were negative by both MI and PCR for three to five days p.i.. Detection by MI took longer than PCR in animal 1, and samples were positive for only three days. In animal 2, MI detected parasite over nine days, although at the periphery of the detection period this was often in only one or two out of three mice inoculated. It was these results which were discrepant with the B1 PCR. The P30 PCR detected parasite over only three days in this animal, whereas the B1 method was positive over seven days.

A total of 101 lymph samples were collected in the period from 4 days prior to infection to 15 days p.i.. The number of positive tests given by MI and the B1 and P30 PCR are shown in Table 3.2. MI detected parasite in 29 samples, whereas the B1 PCR detected 30 positive samples, and the P30 method only 20. All mice which survived inoculation were *T. gondii* IgG negative by ELISA. The mean number of days in which positive results were obtained was 4.8 (SD 3.2) days with MI, 5.0 (SD 1.9) days with the B1 PCR, and 3.2 (SD 2.2) days with the P30 PCR.

Table 3.3 shows how these methods of detecting *T. gondii* compared against each other. The B1 and P30 PCR were compared with MI for each lymph sample. MI detected parasite in 23 samples which were also positive by the B1 PCR. However the P30 PCR detected parasite in only 14 of these samples. The overall reliability of PCR detection compared to MI was 87% using the B1 target and 79% using the P30 target. When frequency of parasite detection by the two PCR methods was compared to only the 29 samples which were MI positive, the agreement between the PCR and MI was 79% for the B1 system and only 48% for the P30 system.

Table 3.1 *T. gondii* detection in efferent lymph by MI, B1 PCR and P30 PCR in animals 1 and 2.

	Animal 1			Animal 2		
Day p.i.	**MI***	**B1**	**P30**	**MI***	**B1**	**P30**
-4	0	-	-	0	-	-
-3	0	-	-	0	-	-
-2	0	-	-	0	-	-
-1	0	-	-	0	-	-
0	0	-	-	0	-	-
1	0	-	-	0	-	-
2	0	-	-	0	-	-
3	0	-	-	1	-	-
4	0	-	-	2	+	+
5	0	+	+	3	+	+
6	0	+	-	3	+	+
7	3	+	+	3	+	-
8	3	+	+	3	+	-
9	3	+	+	3	+	-
10	0	-	-	1	-	-
11	0	-	-	1	-	-
12	0	-	-	0	+	-
13	0	-	-	0	-	-
14	0	-	-	0	-	-
15	0	-	-	0	-	-

* Numbers represent mice (n=3) culled due to acute toxoplasmosis following i.p. inoculation with 250 µl lymph.

Table 3.2 Comparison of MI and the B1 and P30 PCR for detection of *T. gondii* in lymph from infected sheep.

Day p.i.	Number of sheep tested	MI	B1 PCR	P30 PCR
-4	2	0	0	0
-3	4	0	0	0
-2	5	0	0	0
-1	5	0	0	0
0	6	0	0	0
1	6	0	0	0
2	6	0	0	0
3	6	2	1	2
4	6	4	4	5
5	6	4	6	3
6	6	4	5	2
7	6	5	4	2
8	5	4	4	2
9	5	4	3	2
10	5	1	1	1
11	5	1	1	1
12	5	0	1	0
13	5	0	0	0
14	5	0	0	0
15	2	0	0	0
Total	101	29	30	20

Table 3.3 *T. gondii* detection in ovine efferent lymph by the B1 and P30 PCR compared with MI. Data represents combined results from six animals (101 samples).

		B1		P30	
		Positive	Negative	Positive	Negative
MI	Positive	23	6	14	15
	Negative	7	65	6	66

Table 3.4 Detection of *T. gondii* in blood samples from three non-cannulated sheep by the PCR.

		Day p.i.													
Sheep	**PCR**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
A	B1	-	-	-	-	-	+	+	+	+	+	-	-	-	-
	P30	-	-	-	-	-	-	+	+	-	-	-	-	-	-
B	B1	-	-	-	-	-	+	+	+	+	-	-	-	-	-
	P30	-	-	-	-	-	-	+	+	-	-	-	-	-	-
C	B1	-	-	+	-	-	+	+	+	-	-	+	-	-	-
	P30	-	-	-	-	-	-	+	-	-	-	-	-	-	-

3.3.2 Parasite Detection in Blood

In initial studies using blood samples from animal A parasite was detected in five out of nine buffy coat samples, in four whole blood preparations, and in only one boiled preparation. As a result, the buffy coat was used for the remainder of blood samples processed in this study. Preparation methods for human blood samples is investigated in chapter 4.

The results for the blood samples from the three non-cannulated animals are presented in Table 3.4. Parasitaemia was detected earlier with the B1 than with the P30 PCR in all animals. The B1 gene was detected over a mean of 4.7 (SD 0.6) days compared to a mean of 1.7 (SD 0.6) days with the P30 gene ($p=0.003$, 4 df, two-tailed *t* test).

3.3.3 Parasite Detection in Tissue

T. gondii was detected by both the B1 and P30 PCR in the left and right pre-femoral lymph nodes from the sheep killed 12 days p.i.. However samples of liver, spleen, brain, kidney and diaphragm were negative by both methods.

3.4 DISCUSSION

Both the B1 and P30 PCR were capable of detecting *T. gondii* in samples of blood, lymph and tissue from experimentally infected sheep. However the relative sensitivity of these systems differed. Parasite was detected earlier and over a significantly longer period in samples of peripheral blood using the B1 PCR. When compared with MI in lymph samples, parasite detection was significantly higher with the B1 PCR than with the P30 PCR. Discrepancies between MI and the B1 PCR occurred at the periphery of the detection period where parasite numbers were presumably low. A larger sample volume (250 µl) was used to inoculate each of three mice used for every sample, compared to a small 5 µl aliquot from a 50 µl sample preparation for the PCR, therefore sampling error may account for some of the discrepancies seen between MI and PCR. It is possible that accuracy of the test could be improved by repeating the amplification reactions on three occasions, on a similar basis to MI. This would detect the parasite at very low levels, for example when it is present in only one or two out of three aliquots.

In a small number of samples the B1 and P30 PCR detected parasite where MI did not. This may be explained by the ability of the PCR to detect non-viable parasites which would not be identified by MI. Therefore it seems likely that the PCR is capable of detecting parasite DNA from dead organisms after their defeat by the host immune system. The possibility that these results were false positives seems unlikely as none of the large number of negative controls used in each run were positive. In addition, none of the samples of blood or lymph taken before inoculation were positive by either PCR method.

Samples of blood and lymph used in this study were found to be suitable for PCR analysis. However of the various tissue types tested, only lymph node samples produced positive results. As the parasite is known to encyst in a wide variety of different tissue types, it would be likely that experimental infection with such a large number of parasites would increase the chance of detecting DNA from tissue cysts in these animals. Weiss *et al.* (1992) reported that tissue which had been frozen prior to PCR analysis was unreliable when compared to detection in paraffin-embeded tissues for unknown reasons. It is impossible to reject this as a reason for the negative results from most of the tissues tested in this study. However the S48 strain used in this study is an incomplete form of the parasite, only existing as the tachyzoite and is unable to encyst in tissues (Buxton 1990, Buxton *et al.* 1991). It is likely that the PCR amplified DNA from tachyzoites present in the lymph node, or in the lymph fluid itself, while there was no amplification from other tissues because of the lack of tissue cysts in these animals. It is therefore necessary to test other tissue samples from animals infected with different strains of *T. gondii* using a larger number of animals.

The initial hypothesis that the B1 PCR was likely to be more sensitive than the P30 PCR was confirmed and this can be explained by the repetitive nature of the target gene. This was also suggested by Brezin *et al.* (1990) as the reason for the success of the B1 PCR and the failure of the P30 system in the detection of human ocular toxoplasmosis. These results indicate that the B1 PCR should be the system of choice in detecting *T. gondii* in clinical samples, and demonstrates its value as a diagnostic tool.

The PCR has added benefits over MI because of the reduction in time needed to obtain a result from six weeks to one day. This would result in significant changes to treatment regimes in the infected host, with an earlier start to therapy. It also negates the need for large numbers of mice.

CHAPTER 4

T. gondii Infection in the Human Host

4.1 INTRODUCTION

Following the success of the PCR to detect *T. gondii* infection in controlled experimental conditions the techniques were applied to samples from patients. The main part of the study investigated infection in AIDS patients as immunosuppression and illness progressed. However, immunocompetent patients suspected of having toxoplasmosis were also investigated.

AIDS patients are at risk from reactivation of a latent infection as the disease progresses and their CD4+ cell count falls below 100 mm^{-3}. Opinion is currently divided over whether *T. gondii* recrudescence is due to haematogenic spread (Luft and Remington 1992, Dannemann *et al.* 1991), or to local reactivation (Hassl *et al.* 1988, Navia 1986). If reactivation is localised, the chance of detecting parasite using PCR in samples such as blood (and therefore diagnosing infection) is low. However, if the infection is spread through the blood, parasite may be detectable before clinical signs of infection appear. This early detection would be of great value diagnostically, and would also aid the initiation of treatment, which if given early enough, may prevent the development of encephalitis and other illness.

PCR may also be valuable in monitoring the effects of treatment. For example, if initial therapy is successful the dose may be reduced to a maintenance level, with samples then being tested for further signs of recrudescence. It may therefore be possible to reduce maintenance therapy to less toxic levels which would still be capable of preventing relapse.

Published work on the use of PCR for *T. gondii* detection has involved the use of samples such as brain biopsy, CSF and blood (see 1.9.2), but these have involved different methods and complexities of preparation from those investigated here. Blood samples have been treated in a variety of ways, from merely boiling the sample in water to lyse cells (Joss *et al.* 1993, Ho-Yen *et al.* 1992), to proteinase K digestion (van de Ven *et al.* 1991), and also full DNA extraction procedures (Johnson *et al.* 1993). These methods have involved the use of whole blood or buffy coat samples as starting material.

It has been argued that complex sample preparation methods may result in the loss of small amounts of DNA, and that as a result low levels of infection could be missed. Alternatively, simple techniques such as boiling may minimise preparation time and handling, but may have problems with the presence of haemoglobin which is inhibitory to PCR. Several blood preparation methods were therefore investigated.

There were several aims of this study. Firstly to investigate the suitability of different sample types for use in PCR. If blood and urine could be used successfully, they would negate the need for more invasive procedures such as brain biopsy and lumbar puncture. Secondly, preparation methods were investigated with the aim of developing a system which would be both sensitive and involve minimal preparation. The effects of inhibitory factors (Grover *et al.* 1990) were also considered.

4.2 MATERIALS AND METHODS

4.2.1 Patients

HIV infected patients from the Regional Infectious Disease Unit in Edinburgh were enrolled in this study when their CD4+ cell count fell below 100 mm^{-3}, or if toxoplasma encephalitis was suspected on clinical grounds. Patients were assigned to one of three groups and followed prospectively.

Group 1 consisted of individuals with a CD4+ cell count less than 100 mm^{-3} and positive baseline *T. gondii* serology. Group 2 patients had negative *T. gondii* serology and CD4+ cell count less than 100 mm^{-3}, and Group 3 patients had clinical indications of *T. gondii* infection.

Patients in Groups 1 and 2 were identified at each clinic visit. Patients in Group 3, with clinical toxoplasmosis were generally investigated as in-patients and followed up after treatment at each clinic visit.

Patients in other risk groups were also included in this study when toxoplasmosis was suspected, or when it was necessary to exclude toxoplasmosis as a cause of symptoms.

4.2.2 Samples

Samples of blood and urine were taken routinely at each clinic visit, or when toxoplasmosis was suspected. Other samples such as CSF and tissue were also obtained when appropriate as part of the investigation of symptoms. A collection of post mortem samples from HIV positive individuals was also investigated. All samples were tested using B1 and P30 PCR.

4.2.3 Sample Preparation

Blood

Preservative-free heparinised blood was divided into three to five aliquots, depending on the volume obtained. The aliquots used were as follows: (1) 5 ml for white blood cell (WBC) preparations; (2) 100 µl for a boiled preparation; (3) 100 µl for a whole blood (WB) preparation ; (4) 5-10 ml for a Nucleon preparation (Scotlab); (5) Any remaining sample was stored.

A preliminary investigation was carried out to outline the effectiveness of four blood preparation methods. These methods were compared using ten samples from *T. gondii* antibody positive patients, five of whom had clinical toxoplasmosis, and ten samples from *T. gondii* antibody negative patients.

1) Proteinase K digestion of buffy coat (WBC Preparation)

A similar method to that described in 3.2.3 (1) was used where buffy coat was collected from heparinised blood by centrifugation at 800 *g* over lymphoprep (Nycomed, Oslo). Remaining RBC's were removed using TE rather than 10 mM Tris ammonium chloride. This was followed by centrifugation at 13 000 *g* for 30 seconds. The pellet was then resuspended in K buffer containing proteinase K and treated as previously described (3.2.3).

2) Boiled blood preparation

(see 3.2.3).

3) Proteinase K digestion of whole blood (WB Preparation)

This was again similar to the method described in 3.2.3, with TE used to remove RBC's from 100 µl of blood instead of 10 mM Tris ammonium chloride. This was followed by centrifugation at 13 000 *g* for 30 seconds and a further two washes with TE. The pellet was finally resuspended in K buffer containing proteinase K as before.

4) Nucleon preparation

Following the manufacturer's instructions (Scotlab), 5 to 10 ml of heparinised blood was added to 40 ml of reagent A (10 mM Tris, 320 mM sucrose, 5 mM MgCl, 1% Triton x-100 (pH 8.0)). This was agitated at room temperature for four minutes, followed by centrifugation at 1300 *g* for four minutes. The supernatant was discarded and the pellet was resuspended in 2 ml of reagent B (400 mM Tris (pH 8.0), 60 mM EDTA, 150 mM NaCl, 1% SDS), and transferred to a capped 5 ml polypropylene tube. After the addition of 500 µl of 5 M sodium perchlorate the tube was mixed at room temperature for 15 minutes and then at 65°C for 25 minutes. DNA was extracted by the addition of 2 ml of cold chloroform and mixing at room temperature for ten minutes, followed by centrifugation at 800 *g* for one minute. Nucleon silica suspension (300 µl) was added and then the preparation was centrifuged at 1400 *g* for three minutes. The top layer was transferred to a fresh tube and the DNA precipitated by the addition of cold (4°C) 100% ethanol. After centrifugation at 13 000 *g* for ten minutes the pellet was resuspended in 50 µl K buffer.

CSF

Processing was based on the method of Lebech *et al.* (1992). An aliquot of 100 µl of CSF was boiled for 15 minutes using the Techni thermal cycler, after which DNA was precipitated by the addition of 250 µl 100% ethanol at -20°C for one hour. The DNA was collected by centrifugation at 13 000 *g* for 30 minutes, and the resulting pellet washed with 70% ethanol. After further centrifugation the pellet was dried and resuspended in 20 µl of distilled water.

Urine

Urine was divided into two 1 ml aliquots, one was stored and the other was processed as follows. After centrifugation at 13 000 *g*, the pellet was washed three times in saline solution and digested in K buffer containing 100 μg ml^{-1} proteinase K as previously described (3.2.3).

Tissue

Tissue samples were prepared as described in 3.2.3, using TE in place of 10 mM Tris ammonium chloride (pH 7.2) to remove contaminating RBC's.

Post Mortem Samples

All samples were stored at -70°C prior to processing. After thawing, samples were treated as described for tissue above.

All prepared samples were stored at -70°C until testing.

4.2.4 The PCR

B1 and P30 PCR was carried out as described in 2.2.9 - 2.2.11.

4.2.5 Electrophoresis and Southern Blotting

PCR products were analysed on 1-1.5% agarose and then blotted as described in 2.2.14 - 2.2.18.

4.2.6 Statistical Analysis

Statistics were carried out as described in 3.2.6.

4.3 RESULTS

4.3.1 Initial comparison of Blood Preparation Methods

The comparison of DNA extraction methods as a basis for the PCR, using samples from patients is shown in Table 4.1. All methods were used successfully in a PCR to amplify *T. gondii* DNA from at least one sample. Parasite DNA was detected in four WBC, three

WB, five Nucleon and one boiled blood preparations from patients with clinical toxoplasmosis. In patients with latent infection parasite was detected in one WBC preparation and in one Nucleon preparation, and in sample 6 which was negative on repeat testing. Overall the Nucleon system was the most sensitive system with the samples tested. However, of the ten antibody negative patients investigated three were positive using Nucleon. These samples were negative by all other methods, and as there were no signs of toxoplasmosis, these were considered to be false positive results.

The problem with false positive results in the case of the Nucleon system, and false negative results with boiled blood preparations led to the discontinuation of these methods in the remainder of this study.

4.3.2 Detection of Parasite in Samples from HIV Positive Patients

Table 4.2 shows the number of patients studied in each group. Although 31 patients were investigated in Group 1, only 22 attended regularly for follow-up. A similar situation occurred in Group 2, where only 26 out of 51 patients investigated were available for follow-up.

The number and types of samples obtained can be seen in Table 4.3. A total of 221 blood, 19 CSF, 95 urine and five 'other' samples were available for investigation.

Group 1

A total of 104 samples from 22 f.u. patients with antibody to *T. gondii* were tested. The majority of these were negative by B1 and P30 PCR, with the exception of samples from six patients (see Table 4.4). None of these patients had clinical signs of toxoplasmosis.

Group 2

A total of 193 samples were investigated, 142 of which were from the 26 patients followed up in the study. These patients who were *T. gondii* antibody negative, were found to be PCR negative with only two exceptions. Of the two, one patient had

Table 4.1 The PCR results using different blood preparation methods

Sample	Status	PCR	WBC	WB	Nucleon	Boiled
1	Clinical	B1	+	-	+	-
		P30	-	-	-	-
2	Clinical	B1	-	+	+	+
		P30	-	+	+	-
3	Clinical	B1	+	-	-	-
		P30	+	-	-	-
4	Clinical	B1	-	+	+	-
		P30	+	-	-	-
5	Clinical	B1	-	+	+	-
		P30	-	+	-	-
6	IgG +	B1	-	-	+/-	-
		P30	-	-	+/-	-
7	IgG +	B1	-	-	-	-
		P30	-	-	-	-
8	IgG +	B1	-	-	-	-
		P30	-	-	-	-
9	IgG +	B1	-	-	+	-
		P30	-	-	-	-
10	IgG +	B1	+	-	-	-
		P30	-	-	-	-
11	IgG -	B1	-	-	+	-
		P30	-	-	+	-
12	IgG -	B1	-	-	-	-
		P30	-	-	-	-
13	IgG-	B1	-	-	-	-
		P30	-	-	-	-
14	IgG -	B1	-	-	-	-
		P30	-	-	-	-
15	IgG -	B1	-	-	-	-
		P30	-	-	-	-
16	IgG -	B1	-	-	+	-
		P30	-	-	+	-
17	IgG -	B1	-	-	+	-
		P30	-	-	-	-
18	IgG -	B1	-	-	-	-
		P30	-	-	-	-
19	IgG -	B1	-	-	-	-
		P30	-	-	-	-
20	IgG -	B1	-	-	-	-
		P30	-	-	-	-

Table 4.2 HIV positive patients investigated, showing numbers in each group followed, and mean follow-up (f.u.) times.

Group	Status	No. Tested	No. followed-up	Mean time of f.u.
1	*T. gondii* Positive	31	22	3.9 months
2	*T. gondii* Negative	51	26	8.7 months
3	Clinical toxoplasmosis	10	10	10.7 months
Total		92	58	7.8 months

Table 4.3 Samples obtained from the 58 HIV positive patients who were followed-up prospectively.

		Samples			
Group	**Status**	**Blood**	**Urine**	**CSF**	**Other**
1	*T. gondii* Positive	53	45	5	1
2	*T. gondii* Negative	107	25	9	1
3	Clinical toxoplasmosis	61	25	5	3
Total		221	95	19	5

Table 4.4 Samples from Group 1 patients which were PCR positive

Patient	Month fu	Sample	B1	P30
1	1	WBC	-	+
	2	WBC	-	+
2	1	Urine	+	-
3	3	WB	-	+
4	2	WBC	+	-
	2	Urine	+	-
	5	WB	+	-
5	10	WBC	-	+
	10	WB	+	-
6	3	WBC	+	-
	3	WB	+	-

primary toxoplasmosis and so was transferred to group 3 (patient 10), and the other had been tested for antibody at an advanced stage of AIDS, by which time it may have been undetectable. A sample taken five years earlier was located and found to be antibody positive. In this case, samples of CSF (B1 and P30), WBC (P30) and WB (P30) were positive. MRI revealed atrophy of the brain, but no lesions, and CSF contained high levels of protein.

Group 3

PCR results from the 10 patients in group three with clinical signs of toxoplasmosis can be seen in Table 4.5. The mean CD4+ cell count of this group was 8 mm^{-3}. PCR detected parasite in blood samples in 11 of 13 episodes of toxoplasmosis. Several patients suffered from repeated recrudescence for example in patients 2, 3 and 9. In three cases (patients 2, 3 and 10), parasite was detected in blood samples up to four weeks prior to

clinical signs of infection. An example of progressive infection can be seen in Table 4.6 (patient 2).

Urine samples were positive in patients 3 and 8. These were positive at advanced stages of toxoplasmosis, often after symptoms had been controlled.

Only one of the five CSF samples tested was positive by PCR (patient 2), even though they were all obtained during clinical toxoplasmosis.

Brain biopsy was carried out on two patients (1 and 5) and these were both positive. Blood from patient 1 was also PCR positive, however no other samples were available at this time from patient 5.

Patient 9 was an infant with an apparent primary infection. Both parents were *T. gondii* IgG negative and HIV positive. MRI revealed lesions and blood samples were PCR positive, which confirmed the diagnosis. The patient improved clinically when treatment commenced. However as the treatment was reduced, recurrence of symptoms occurred and *T. gondii* DNA was again detected by the PCR in blood samples. Patient 10 was originally *T. gondii* antibody negative, but was found to have primary toxoplasmosis. Throughout this period there was no evidence of IgG or IgM, however response to treatment was good.

T. gondii DNA was not detected in blood, urine or CSF from patient 4, although PM samples tested were positive by the PCR. This case will be discussed later (4.4).

Table 4.7 shows the success rate of B1 and P30 PCR in the detection of parasite in samples from patients in group 3 who had confirmed toxoplasmosis. In the 19 samples tested, B1 PCR using WB preparations detected 63.2% of positive samples, and 47.1% using WBC preparations. P30 detected 47.4% of WB samples, and 41.2% of WBC samples. These were not statistically different ($p=0.581$).

Table 4.5 Summary of clinical details and PCR results from patients in Group 3 with toxoplasmosis.

PATIENT	SEX	AGE	CLINICAL DETAILS	CD4	TOXO IgG	PCR	BLOOD	WBC	URINE	CSF	TISSUE
1 (IDU)	M	36	Confusion, haemiparesis	0	+	B1	+	-	0	0	+
			CT scan abnormal +			P30	+	-	0	0	+
2 (IDU)	M	28	Painful swallowing	3	+	B1	+	+	-	0	0
Episode 1			MRI scan abnormal ++			P30	+	+	-	0	0
Episode 2			Fit	4		B1	+	-	-	+	0
						P30	+	-	-	-	0
3 (H0)	M	33	(2 prior episodes) Convulsions	9	+	B1	+	-	0	-	0
Episode 3			CT scan normal			P30	+	-	0	-	0
Episode 4			Convulsions	3		B1	-	+	+	0	0
			MRI scan abnormal ++			P30	-	+	+	0	0
4 (Bi)	M	45	(1 prior episode)	10	+	B1	-	-	-	-	+
Episode 2			MRI scan abnormal +			P30	-	-	-	-	+
5 (Ho)	M	41	(1 prior episode)	4	+	B1	0	0	0	0	+
Episode 2						P30	0	0	0	0	+
6 (IDU)	M	30	Deafness, nephritis	14	+	B1	+	+	0	0	0
			hypertension			P30	-	+	0	0	0
7 (IDU)	M	32	Ataxia	10	+	B1	+	+	+	0	0
			Vomiting			P30	+	+	+	0	0
8 (HET)	M	65	MRI scan abnormal +	8	+	B1	+	-	0	0	0
			CVA			P30	-	+	0	0	0
9 (VER)	F	9/12	Fits	NA	-	B1	+	+	0	-	0
Episode 1			MRI scan abnormal +			P30	-	-	0	-	0
Episode 2			Treatment reduced			B1	+	0	0	0	0
			Encephalitis			P30	+	0	0	0	0
10 (IDU)	F	31	MRI scan abnormal ++	0	-	B1	+	+	0	0	0
						P30	+	-	0	0	0

Table 4.6 Recrudescence in patient 2 (Group 3).

Date	Sample	B1	P30	CD4	Clinical Data
3/93	WBC	-	-	9	Treatment: Dapsone.
	WB	-	-		
	Urine	-	-		
4/93	WBC	+	+	3	Episode 1
	WB	-	+		Cerebral mass lesions
5/93	Urine	-	-	4	Lesions reduced
	Urine	-	-		
6/93	WBC	-	-		
	WB	+	-		
8/93	CSF	+	-		Episode 2
	WBC	-	-		Treatment: Pyrimethamine
	WB	+	+		and Sulphadiazine
8/93	WBC	-	-		
	WB	-	-		
	Urine	-	-		
9/93	WBC	-	-		
	WB	-	+		
10/93	WB	-	-		
10/93	WBC	-	-		
	WB	-	-		

Table 4.7 Positivity rates of the B1 and P30 PCR in WBC and WB preparations

	Blood Preparation Method	
PCR	**WBC**	**WB**
B1	8/17* (47.1%)	12/19 (63.2%)
P30	7/17* (41.2%)	9/19 (47.4%)

* Two samples were insufficient for WBC preparation.

Table 4. 8 PCR results from PM samples from *T. gondii* antibody positive patients

Sample	No. tested	B1 PCR Positive	P30 PCR positive
CSF	3	1	0
Brain	8	4	4
Spinal cord	7	4	4
Lymph node	6	1	2
Thymus	6	2	2
Liver	6	1	0
Lung	5	1	0
Bone marrow	6	1	1
Adrenal	6	1	0
Spleen	6	1	0
Colon	6	1	0
Kidney	7	0	0
Myocardium	5	2	3
Pancreas	3	1	0
Rectal muscle	3	1	1

Post Mortem Study

PM samples were obtained from ten patients, eight which were antibody positive, and two which were antibody negative. Three of the PM's were small, containing only brain and spinal cord, however most included up to 15 tissues i.e. brain, spinal cord, adrenal, thymus, myocardium, diaphragm, lung, kidney, liver, pancreas, colon and rectal muscle. A total of 19 samples were tested from the two *T. gondii* antibody negative patients, one of whom had been followed in group 2. All of these were B1 and P30 PCR negative.

Of the eight *T. gondii* antibody positive PM's, one containing only three samples was PCR negative. The remaining seven had at least one tissue which was PCR positive. A summary of PM results is presented in Table 4.8. Samples (n=15) obtained at PM examination from patient 4 (see Table 4.5) in group 3, who suffered from clinical toxoplasmosis, were tested. Although samples collected at the time of illness were negative by PCR, 14 PM samples were positive by the B1 PCR, and seven were positive by P30 PCR.

4.3.3 Detection of Parasite in Samples from HIV Negative Patients

Eight patients with suspected toxoplasmosis were investigated. Fifteen blood, six urine, three CSF and placenta, amniotic fluid, cord blood and skin biopsy samples were available. Five of the patients were *T. gondii* positive by PCR (patients 3, 4, 5, 6 and 8) in a wide variety of samples which are outlined in Table 4.9. Patient 1, with leukaemia was tested in order to exclude toxoplasmosis as a cause of symptoms. Patient 2 accidentally inoculated himself with live parasite during an experimental procedure. He was treated immediately and blood taken at the time was PCR negative and *T. gondii* antibody positive, showing signs of previous infection. Patient 3 contracted primary toxoplasmosis during the last trimester of pregnancy. Blood was found to be PCR positive at this time and the patient was given standard treatment. Samples of blood, amniotic fluid, placenta and cord blood were also found to be positive at delivery. The infant appeared to be normal at this stage, and also when examined several months later. Patient 4, who was *T. gondii* antibody positive became ill several months after receiving a kidney transplant. She responded to treatment and remains well, although one blood

sample taken three months later was still positive by the PCR. Patient 5 was in a high risk group because of intravenous drug use. He was found to have a primary infection and samples remained PCR positive over three months. Three patients were not immunocompromised, but toxoplasmosis was suspected on the grounds of lymphadenopathy (patients 6, 7 and 8). Two of these were primary infections, with samples remaining positive by the PCR for four months for patient 6 and two months for patient 8.

Table 4.9 PCR results using samples from HIV negative patients with suspected toxoplasmosis.

Patient	PCR	WBC	WB	Urine	CSF	Other
1) Leukaemic	B1	-	-	0	-	0
	P30	-	-	0	-	0
2) Needlestick	B1	-	-	0	0	0
	P30	-	-	0	0	0
3) Pregnant	B1	+	+	0	0	Amniotic fluid, cord
	P30	+	+	0	0	blood & Placenta +
4) Transplant	B1	+	-	0	-	0
	P30	+	-	0	-	0
5) IVDU	B1	-	+	+	0	0
	P30	-	+	-	0	0
6) Child (4 yr)	B1	+	+	0	0	0
	P30	+	+	0	0	0
7) Child (10 yr)	B1	0	-	0	-	0
	P30	0	-	0	-	0
8) Female	B1	+	-	+	0	Skin biopsy -
	P30	-	-	+	0	

4.4 DISCUSSION

Initial work on the preparation of blood samples for use in PCR demonstrated that the simple technique of boiling samples in water was insensitive, and revealed that the more complex Nucleon system was prone to contamination problems. The problem with the latter was due to the number of steps involved, each of which increased the chance of contamination with *T. gondii* DNA, even though the number of steps was less than in a traditional DNA extraction (2.2.3). The method found to be the most useful for the detection of parasite was proteinase K digestion which is a compromise between the simple boiling method, which may not break down cellular material sufficiently to release DNA, and the Nucleon system. Proteinase K was also found to be more efficient than the boiling system in the preparation of experimental mock samples (Groß *et al.* 1992).

The preparation of other samples such as tissue and CSF became more documented during the course of this study (Lebech *et al.* 1992, Gozlan *et al.* 1992) and so comparison of different methods was not carried out. However, because of indications of the inhibitory effects of cellular debris (Grover *et al.* 1990), a small amount of starting material was used in tissue preparation and this was standardised at 0.2 g throughout the study.

In AIDS patients, blood samples were found to be useful in the diagnosis of toxoplasmosis, often before clinical systems were apparent. Whole blood preparations were more sensitive than WBC preparations, however similar results were not obtained from immunocompetent patients. There may be two explanations for this; one possibility is that the majority of parasite is present in whole blood samples, and not in the WBC fraction (Hitt and Fillice 1992); secondly, in an immunocompetent individual a large number of cells can be obtained from the buffy coat, and therefore a small number of parasites can be detected. In AIDS patients where the WBC count is severely reduced (see Table 4.5) it is often difficult to see any band in the layer over the Lymphoprep. Therefore most parasite may be lost to the red blood cell (RBC) layer, and not detected by the PCR.

It was also possible to detect parasite from urine samples, but these were generally positive later than blood samples, and often after illness had subsided. This may have been due to the detection of DNA from non-viable parasites which had been broken down by therapeutic agents or the immune system, and were being cleared by the kidney. This suggests that these samples are of little practical use in a diagnostic setting.

Parasite was detected in both brain biopsy samples obtained. No other samples were available at this time from one of these patients (patient 4), and PCR along with MRI and clinical findings produced a diagnosis. However blood samples from the other patient were positive. If blood had been taken initially, a positive PCR result would have made the invasive procedure unnecessary.

The combination of placenta and amniotic fluid was useful in the diagnosis of congenital toxoplasmosis. Only two ocular samples were obtained which were negative, and so it is not possible to speculate how useful these samples may be in the diagnosis of ocular *T. gondii* infection.

Disappointingly, CSF proved to be unhelpful in the diagnosis of toxoplasmosis, with only one positive PCR result. This was not due to inhibitory factors in most cases, as few of these samples prevented amplification in control reactions carried out alongside the test.

Parasite was detected in blood, often before illness, which suggests that recrudescence is not merely a localised event in the brain, but that parasite is also transported via the blood to target organs. Therefore testing blood samples by the PCR is useful in the diagnosis of infection in patients where more traditional serological techniques are inadequate. These include AIDS patients, transplant patients, pregnant women and their new-born infants as demonstrated in this study. In these cases serology would have limited applications because of maternal antibody in the case of congenital toxoplasmosis, and the prior existence of antibody in the transplant patient.

There are some limitations associated with the PCR in the diagnosis of toxoplasmosis in AIDS patients. Parasite was found circulating in 27.2% of *T. gondii* antibody positive patients on at least one occasion during this study. These patients showed no clinical signs of toxoplasmosis, which may mean that the system detected the sporadic breakdown of tissue cysts. The mean CD4+ cell count among these individuals was 46 mm^{-3}, which was higher than those with clinical toxoplasmosis with a mean of 8 mm^{-3}. It is difficult to assess whether there were no clinical indications in these cases because the individuals still had some immunological capacity to destroy parasite released from the sporadic breakdown of cysts and so prevent recrudescence, or because of some other genetic factor. This was suggested by McLeod *et al.* (1989), and Suzuki *et al.* (1991) as a reason for the ability of certain strains of mice to prevent infection.

Parasite was detected in samples from seven out of eight PM's from antibody positive patients, but only 50% of CNS samples (such as brain and spinal cord) were found to contain parasite. In comparison the CNS was involved in all ten cases of recrudescence in group 3. As the PCR can detect tissue cysts, these could not be present in large numbers in patients with chronic infection as they were not always detected. This suggests that parasite can invade the CNS from other sources to some extent during reactivation. Therefore blood may transport the parasite to the brain and be responsible for many, but not necessarily all, cases of recrudescence. One PM was from a patient with clinical toxoplasmosis from which strong positive results were obtained from most tissues (14 out of 15). However with this PCR system it is impossible to differentiate active infection from the detection of latent tissue cysts.

In conclusion, the success of blood as a sample in which to detect parasite makes invasive techniques such as brain biopsy and lumbar puncture unnecessary. However not all cases of recrudescence were detected by PCR in these samples. The timing of sample collection is therefore believed to be critical to successful diagnosis. Results should be taken into consideration with clinical and MRI details. There is therefore a need to further investigate parasite / host interaction.

CHAPTER 5

The Development of an Animal Model of Recrudescence of *T. gondii* Infection

5.1 INTRODUCTION

The incidence of recrudescence of toxoplasmosis in AIDS patients with evidence of past infection is reported to be 33% (Luft and Remington 1992). Therefore many of these patients are at risk in countries such as France where 80% of individuals have IgG against *T. gondii* (Wanke *et al.* 1987).

In the patient study (chapter 4), 15% of the Edinburgh AIDS cohort was found to have antibody to *T. gondii*. Parasite was detected by the PCR in peripheral blood, often before clinical manifestations in these patients. This suggests that a parasitaemia is involved in reactivation although it may be transient. However parasite was detected in six AIDS patients with *T. gondii* IgG (Group 1), where there were no clinical signs of reactivation.

The aim of this study was to develop an animal model of recrudescence to investigate the significance of parasitaemia in patients at risk. This may provide information on the incidence of parasitaemia, and its relevance as an indicator in recrudescence from latent to acute infection. The number of animals affected by recrudescence could be monitored and various samples investigated for evidence of infection.

A limited amount of work has been carried out using animals to investigate parasite / host interaction during acute infection (Conley and Jenkins 1981, Ferguson and Hutchison 1987). Studies involving the use of immunocompromised mice include the use of nude mice by Lindberg and Frenkel (1977). Results revealed that nude mice died 6.3 days post infection, unless they received transplanted thymic cells. However the need for a good animal model to investigate new drug therapies was highlighted by Johnson (1992). This must therefore involve animals with recrudescence of a chronic infection. Lymphodeficient SCID mice were used by Johnson (1992) to investigate chronic infection, however both nude and SCID mice are difficult to work with because of their severely impaired immune systems, and require isolation units and sterile conditions. To avoid these complications the method chosen for this study was similar to that described by Powles *et al.* (1992) where immunosuppression and *Pneumocystis carinii* infection resulted from the addition of dexamethasone to the animals' drinking water. This would

enable the establishment of a chronic *T. gondii* infection in mice prior to dexamethasone treatment and therefore mimic the situation in AIDS patients where a chronic infection is established prior to immunosuppression.

5.2 PRELIMINARY INVESTIGATIONS

5.2.1 Pilot study

A pilot study was carried out to investigate recrudescence in chronically infected mice treated with dexamethasone with the aim of mimicking reactivation of infection in the immunocompromised human host. If successful, this animal model would be a valuable tool in studying the mechanisms involved in the switch from latent to active infection.

Experimental Design

Porton mice were chronically infected following i.p. inoculation with 30 *T. gondii* tissue cysts of the M3 isolate (groups 1 - 3). Groups 4 - 6 consisted of *Toxoplasma* negative mice. Details of treatment protocol is shown in Table 5.1.

Table 5.1 Treatment protocol during the pilot study

Group	no.	*Toxoplasma* status	Dexamethasone level
1	9	Chronic Infection	8 mg l^{-1}
2	9	Chronic Infection	4 mg l^{-1}
3	10	Chronic Infection	nil
4	10	Negative	8 mg l^{-1}
5	9	Negative	nil
6	10	Negative	8 mg l^{-1} + Tetracycline (1 g l^{-1})

Six weeks after the initial infection dexamethasone was administered in the drinking water of the appropriate groups which was then changed every two days. Blood samples were taken by tail bleed before immunosuppression treatment commenced, and weekly thereafter. Mice were monitored daily over eight weeks, and animals which showed signs of illness were culled using excess CO_2. Samples of brain, blood and heart were taken for PCR analysis. Signs of illness included limited "clockwork" mobility, and a hunched appearance alongside evidence of emaciation and dehydration. At the end of the eight week study period all remaining mice were culled and examined as above. All samples were examined using the B1 PCR.

Results

At the higher level of dexamethasone treatment (Group 1) 6/9 mice showed signs of recrudescence within the eight week test period. In comparison, only 3/9 mice on low level dexamethasone treatment (Group 2) became ill due to reactivation of infection. None of the chronically infected mice in group 3 which did not receive dexamethasone treatment showed any sign of illness. This was also the case with mice from Groups 4, 5 and 6, where none of the samples tested were PCR positive. However some mice negative for *Toxoplasma* (Group 4) became ill due to infection from unrelated opportunistic organisms. This problem was eliminated by Tetracycline administered at levels of 1 g l^{-1} in drinking water (Group 6).

The pilot study indicated that dexamethasone was appropriate in the development of a mouse model of *T. gondii* recrudescence. Therefore the study was repeated with the inclusion of pathological and serological investigations which could reveal useful information on the recrudescence of *Toxoplasma* in the immunocompromised host. This study may indicate whether the PCR detected the presence of tachyzoites (which indicate an active infection), or merely detected sporadic tissue cysts, or products from their breakdown. Serological testing and pathological examination may also be of value in recording how antibody levels and parasite recrudescence vary with immunosuppressive therapy.

5.3 MATERIALS AND METHODS

5.3.1 Treatment Protocol

A pool of 108 chronically infected Porton mice was established (54 females and 54 males), and a further 50 naive Porton mice (25 females and 25 males) were also included as control groups. Data from the pilot study indicated that to observe differences between treatments at the 5% significance level it was necessary to include 20 mice in each group. Details of the treatment protocol can be seen in Table 5.2. All groups which received dexamethasone also received 1 mg l^{-1} tetracycline in drinking water for the duration of the study. In addition to the 100 mice used in this part of the study, a further two from Groups 1 to 3 were used each week to monitor the progression of infection in chronically infected mice (16 per group over eight weeks). In Group 4, a *Toxoplasma* negative control, only one animal was tested weekly. These were sequentially culled and tested for evidence of recrudescence.

Table 5.2 Treatment protocol

Group	*Toxoplasma* status	Dexamethasone level	No. for main study	No. for sequential testing
1	Chronic Infection	8 mg l^{-1}	20	16
2	Chronic Infection	4 mg l^{-1}	20	16
3	Chronic Infection	nil	20	16
4	Negative	8 mg l^{-1}	20	8
5	Negative	nil	20	2

5.3.2 Investigations

Mice were observed daily, and samples of blood, brain and heart were collected from culled mice as described in the pilot study (5.2.1). The rate of recrudescence was assessed by the number of mice showing signs of illness which were culled and investigated.

PCR Analysis

The B1 PCR was used to examine samples of blood, brain and heart obtained at PM examination, and also weekly blood samples.

Pathology

Brain samples were obtained from all mice at PM examination and were fixed in 10% formal saline before embedding in paraffin wax. Four 5 μm sections were cut and stained with HE then examined for evidence necrosis, gliosis and lymphoid inflammation. Sections in which no evidence of change was found were scored (-); sections with minimal to mild presence of change was found were scored (+); and those with moderate to severe change were scored (++). (Work carried out by S. Maley and D. Buxton).

Serology

Plasma samples were tested for IgG against *T. gondii* using the ELISA (Buxton *et al.* 1988) on day 0, and then again at PM examination (work carried out by S. Maley).

Statistical Analysis

Statistical analysis was performed on the effect of dexamethasone on the presence or absence of the parasite in samples, and also on the mortality rate in the different treatment groups. Analysis was carried out using Fischer's Exact test which is unaffected by low or zero counts.

5.4 RESULTS

5.4.1 Level of Recrudescence

The results of the B1 PCR using samples of blood, brain and heart can be seen in Table 5.3. The mortality rate was 36.4% (8 out of 22) in Group 1, 10% (2 out of 20) in Group 2, and 0% in Group 3. One animal died in Group 4, but this was accounted for by the occasional loss of animals in any given group. This animal had no symptoms suggestive of toxoplasmosis, but unfortunately no samples were available for testing because most of the animal had been consumed by other members of the group. Statistical analysis revealed that the mortality rate of animals in Group 1 was significantly higher than that of Group 3 ($p=0.0027$), and of borderline significance when compared to Group 2 ($p=0.0485$). The mortality rate in Group 2 was not significantly higher than Group 3 ($p=0.2436$).

In the 10 mice from Groups 1 and 2 which showed signs of recrudescence, parasite was detected in 9 brain samples, 6 heart samples, and 3 blood samples (only 8 were available for testing).

5.4.2 Weekly Blood Samples

The results of the B1 PCR analysis of the weekly blood samples are shown as percentages in Table 5.4 as numbers decreased with time. Samples were positive in 38.9% (49 out of 126) samples from Group 1, 23.4% (32 out of 137) samples from Group 2, and 8.6% (12 out of 140) samples in Group 3. These results were highly significant: Group 1 against Group 2 ($p=0.0064$); Group 1 against Group 3 ($p<0.0001$); Group 2 against Group 3 ($p=0.008$). All samples from Groups 4 and 5 were negative.

5.4.3 Sequential Culls

None of the samples obtained from Groups 4 and 5 was positive by the B1 PCR (see Table 5.5). In contrast, most brain samples were positive in Groups 1 to 3, with no difference between treatment regimes. None of the blood samples was PCR positive in Group 3, whereas 3 out of 10 were positive in Group 2, and 6 out of 10 were positive in

Table 5.3 The B1 PCR results using samples from mice showing signs of recrudescence

Group	D14	D15	D17	D18	D19	D20	D21	D31	D32	D34	D38	LOST
1 T+ 8 mg		Bl + Br + H +	Bl - Br + H +	Bl 0 Br + H -	Bl + Br + H -	Bl - Br + H -	Bl + Br - H +	Bl - Br + H +		Bl - Br + H +		36.4%
2 T+ 4 mg	Bl 0 Br + H +										Bl - Br + H -	10%
3 T+ 0 mg												0%
4 T- 8 mg									Bl 0 Br 0 H 0			5%
5 T- 0 mg												0%

0 - No sample available

Bl - Blood

Br - Brain

H - Heart

Table 5.4 Numbers of weekly blood samples which were positive by the B1 PCR (shown as a percentage because of reducing numbers).

Group / Treatment	D0	D7	D14	D21	D28	D35	D42	MEAN
G1 (T+ 8 mg)	10	30	55 (-5)	35 (-1)	43 (-2)	72	33	38.9
G2 (T+ 4 mg)	5	30 (-1)	25	20	30	27	27	23.4
G3 (T+ 0 mg)	0	15	10	5	10	10	10	8.6
G4 (T- 8 mg)	0	0	0	0	0 (-1)	0	0	0
G5 (T- 0 mg)	0	0	0	0	0	0	0	0

(Numbers in brackets refer to mice lost during the preceding week)

Group 1. Group 1 was significant against Group 3 (p=0.0054), but not against Group 2 (p=0.1849). Heart samples were positive by the PCR in 7 out of 10 cases in Groups 1 and 2, and in only 1 out of 10 cases in Group 3, which was significantly different (Group 1 against Group 3, and Group 2 against Group 3 p=0.0099).

5.4.4 Final Culls

The results of the B1 PCR using samples from PM examination at the final cull is presented in Table 5.6. Blood was positive by the B1 PCR in 5 out of 14 (35.7%) samples from Group 1, 5 out of 18 (27.7%) in Group 2, and 3 out of 20 (15%) in Group 3. These results were not significantly different. All brain samples were positive in Group 1, compared with 17 out of 18 (94.4%) in Group 2, and 15 out of 20 (75%) in Group 3. These results were not significantly different. A similar result was obtained with heart samples which were positive in all 14 samples in Group 1, 15 out of 18 (83.3%) in Group 2, and 9 out of 20 (45%) in Group 3. There was a significant difference between Groups 1 and 3 (p=0.0006).

5.4.5 Pathology

Haematoxylin / eosin staining of brain sections demonstrated the effects of dexamethasone on necrosis, gliosis and lymphoid inflammation, in treated and control animals (see Table 5.7 and Figures 5.1 to 5.4).

Necrosis

Dexamethasone increased the level of necrosis in Groups 1 and 2, especially in mice showing clinical signs of illness (Figure 5.3). The level of necrosis was low in apparently well animals when compared to those that were ill (Table 5.7b).

Gliosis

The effect of dexamethasone was to reduce the incidence of gliosis in most animals. However gliosis was present in mice which were ill when compared to those which were well (Figure 5.2) (Table 5.7b).

Table 5.5 The B1 PCR results from PM examination of sequentially culled mice.

Group	Sample	Positive	Negative
Base Line	Blood	0	2
T. gondii IgG positive	Brain	2	0
(day 0) (n=2)	Heart	1	1
Group 1	Blood	6	4
(n=10)	Brain	9	1
	Heart	7	3
Group 2	Blood	3	7
(n=10)	Brain	9	1
	Heart	7	3
Group 3	Blood	0	10
(n=10)	Brain	9	1
	Heart	1	9
Group 4	Blood	0	5
(n=5)	Brain	0	5
	Heart	0	5
Group 5	Blood	0	2
(n=2)	Brain	0	2
	Heart	0	2

Table 5.6 The B1 PCR results from samples taken at PM examination at the final cull.

Group	Sample	Positive	Negative
1	Blood	5	9
(n=14)	Brain	14	0
	Heart	14	0
2	Blood	5	13
(n=18)	Brain	17	1
	Heart	15	3
3	Blood	3	17
(n=20)	Brain	15	5
	Heart	9	11
4	Blood	0	20
(n=20)	Brain	0	20
	Heart	0	20
5	Blood	0	10
(n=10)	Brain	0	10
	Heart	0	10

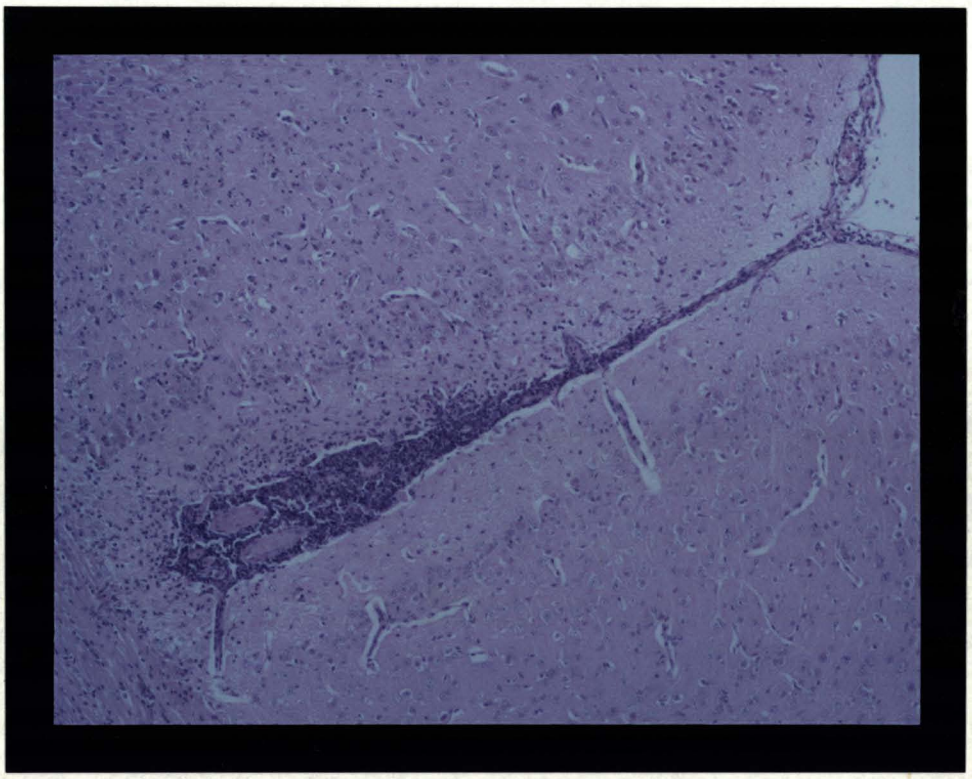
Table 5.7a Results (%) of pathological examination of brain sections (total numbers).

	Necrosis			Gliosis			Lymphoid Inflammation		
Group	-	+	++	-	+	++	-	+	++
1 (n=32)	84	13	3	41	50	9	9.5	87	3.5
2 (n=31)	97	0	3	39	55	6	9	75	16
3 (n=30)	100	0	0	23	67	10	0	70	30
4 (n=25)	100	0	0	100	0	0	100	0	0
5 (n=10)	100	0	0	100	0	0	100	0	0

Table 5.7b Results (%) of pathological examination of brain sections. Numbers in brackets refer to those mice showing clinical signs of illness in Groups 1 and 2.

	Necrosis			Gliosis			Lymphoid Inflammation		
Group	-	+	++	-	+	++	-	+	++
1 n=24	92	8	0	46	46	8	12.5	87.5	0
(n=8)	(57)	(33)	(10)	(20)	(70)	(10)	(0)	(90)	(10)
2 n=29	100	0	0	41.5	55	3.5	10	76	14
(n=2)	(50)	(0)	(50)	(0)	(50)	(50)	(0)	(50)	(50)
3 n=30	100	0	0	23	67	10	0	70	30
4 n=25	100	0	0	100	0	0	100	0	0
5 n=10	100	0	0	100	0	0	100	0	0

Figure 5.1 Lymphoid meningitis in the median sulcus of the cerebrum.



Cage 4 (T+ 4 mg l^{-1})

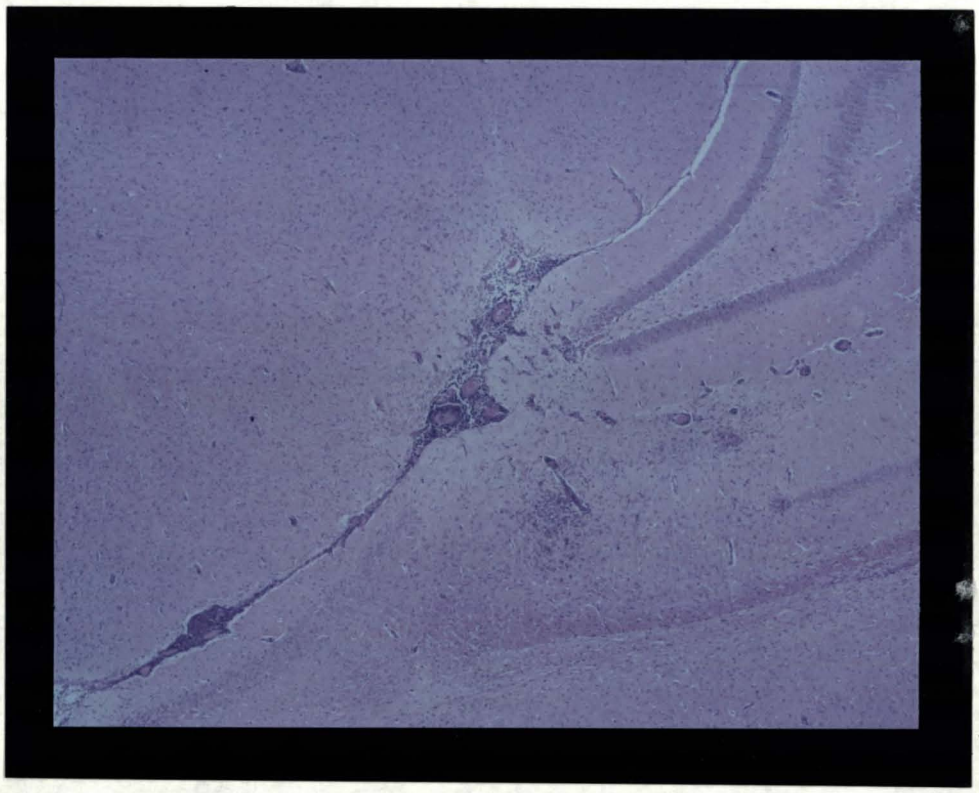
Mouse 1

HE staining

x10

Photograph by D. Buxton

Figure 5.2 Focal gliosis and mild lymphoid perivascular cuffing adjacent to the hippocampus



Cage 4 (T+ 4 mg l^{-1})

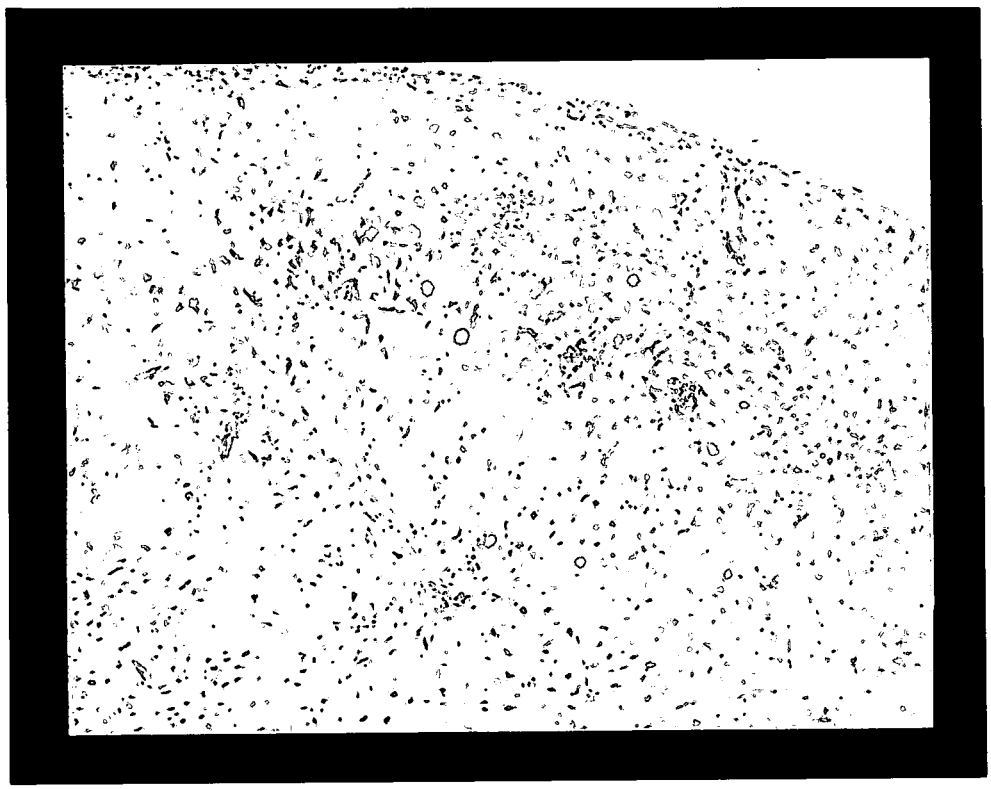
Mouse 1

HE staining

x10

Photograph by D. Buxton

Figure 5.3 Acute necrosis and numerous *T. gondii* tissues cysts in the cerebral cortex.



Cage 3 (T+ 4 mg l^{-1})

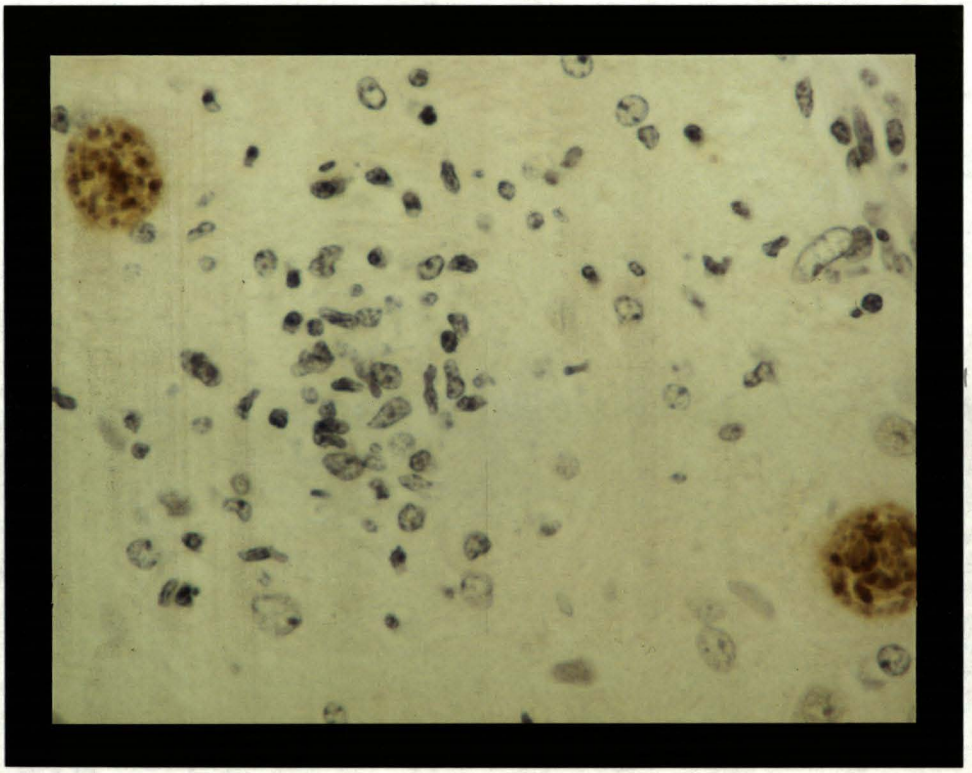
Mouse 8

HE staining

x10

Photograph by D. Buxton

Figure 5.4 Two *T. gondii* tissue cysts with a small associated glial focus.



Cage 5 (T+ nil mg l^{-1})

Mouse 6

Immunoperoxidase staining

x20

Photograph by D. Buxton

Lymphoid Inflammation

The incidence of lymphoid inflammation was reduced with dexamethasone treatment when the total number of mice was observed. Again, when levels were compared between sick mice and well mice, the level of lymphoid inflammation was higher in those which were ill (Table 5.7b).

5.4.6 Serology

Results of the IgG ELISA on the blood samples obtained at day 0 were compared with those obtained at PM examination (see Table 5.8). At day 0, all samples from mice in Groups 1 to 3 were positive, with similar OD values. Samples from mice in Groups 4 and 5 were negative. Samples from Groups 1 to 3 remained positive after treatment, however the mean OD value dropped from 121.5 to 99.5 in Group 1.

Table 5.8 IgG ELISA results from mice on day 0 and day 42.

Group	No. tested (day 0)	OD. Value	No. tested (Day 42)	OD. Value
1	12	121.5	14	99.5
2	12	119.7	18	119.7
3	12	128.6	20	126.7
4	8	10*	20	10*
5	8	10*	10	10*

*Cut off = 25

5.5 DISCUSSION

Previous studies of *T. gondii* infection in animals have revealed a lot about parasite / host interaction. However many areas such as CNS involvement remain unclear. Tissue cysts in the brain remain within intact neurones which explains the absence of an inflammatory response (Ferguson and Hutchison 1987), but this differs from the cases of recrudescence in this study where an inflammatory response was seen in sick animals (5.4.5). A similar response was documented by Vollmer *et al.* (1987) where neurologic symptoms were present alongside symptoms of dehydration and emaciation.

Results in a study by Johnson (1992) using SCID mice revealed that CD4+ CD8+ T cells are crucial to the development of immunity in the host. However despite a deficiency of T and B lymphocytes, SCID mice probably possess another mechanism of temporary resistance to active *T. gondii* which is gamma interferon-dependent. This did not protect animals for long as they all succumbed to infection within a short time (mean of 6.3 days). The use of SCID mice and monoclonal antibody was rather artificial when compared to recrudescence in the AIDS population where only 30 - 35% of individuals with chronic infection show signs of infection. Although Johnson (1992) suggests that drugs such as cortisone may be non-specific, drug therapy based on the methods of Powles *et al.* (1992) was chosen for this study to better mimic the effects seen in the AIDS population.

5.5.1 The Use of Dexamethasone to Create an Animal Model of *T. gondii* Recrudescence

Dexamethasone was successfully used to create a mouse model of *Pneumocystis carinii* infection (Powles *et al.* 1992). The application of dexamethasone resulted in an increased mortality rate in chronically infected mice, especially at the high dose rate of 8 mg ml^{-1} (Group 1). These animals exhibited classic locomotor signs of toxoplasmosis which were not observed in any of the control groups. There were no differences in the detection of parasite in samples of blood and brain in Groups 1, 2, and 3, but heart samples were positive on a greater number of occasions when compared to untreated mice (Group 3). Serological testing also demonstrated a decrease in the OD value the of IgG ELISA in

Group 1, indicating that dexamethasone did cause immunosuppression at a high level of administration.

The results of pathological examination of brain sections reveal two effects of dexamethasone on the mechanics of infection. Firstly, dexamethasone acts as immunosuppressant and therefore causes recrudescence of a chronic infection in a number of mice. Secondly, the drug also acts as an anti-inflammatory agent (and is often used as such), and so reduced the amount of damage in many cases. Levels of necrosis were not influenced by the anti-inflammatory effects of the drug and so the overall result was an increase in damage with increased levels of therapy. However the level of gliosis and lymphoid inflammation was influenced by anti-inflammatory effects and so reduced any pathological symptoms. This was not the case in sick mice where the majority showed moderate to severe signs of both gliosis and lymphoid inflammation.

These results indicate that dexamethasone did cause recrudescence in chronically infected mice, and is a useful tool for further investigation of parasite / host interaction. However there may be problems due to the anti-inflammatory effects of the drug which could influence the level of recrudescence in treated animals.

5.5.2 The Incidence of Parasitaemia in Recrudescence of *T. gondii* Infection

The detection of parasite in the blood of chronically infected mice from day 0 implies that parasite does occasionally circulate in the host, as was suggested by Conley and Jenkins (1981). In most cases this is cleared by the immune system to prevent recrudescence. Immunosuppression resulted in an increase in the number of samples in which parasite was detected, however these results were not always associated with clinical signs of recrudescence during the course of the study. The samples may be positive by the PCR due to intermittent cyst breakdown and the release of small numbers of parasite into the blood which are not high enough to cause recrudescence, or be inactivated by any remaining defences within the host. This observation is backed up by the occurrence of positive samples obtained from chronically infected mice with no immunosuppressive therapy (Group 3).

5.5.3 The Use of Blood Samples for the Detection of Recrudescence

As parasite was often detected in mice which remained clinically well, blood samples may be of limited value in the diagnosis of recrudescence of chronic infection in the immunocompromised host. However they may still be of use when considered alongside other sampling and testing protocols. This was also the case in the patient study where blood samples were positive by the B1 PCR in a number of cases (Chapter 4). These were unlikely to be false positive results because none of the many negative controls used throughout each test were positive, and the control animals remained negative throughout the study. A positive result from a blood sample from an individual at risk may indicate a high risk of recrudescence in the near future, and suggests the need for close monitoring for symptoms of toxoplasmosis.

Other samples such as heart may be useful in detecting recrudescence as parasite was detected in samples from immunosuppressed mice in a significant number of samples. Brain samples tended to be positive in most chronically infected mice, whether they received immunosuppressive therapy or not. Therefore, although brain biopsy has been used to detect many cases of recrudescence, the PCR may be detecting high levels of tissue cysts rather than active tachyzoites. In addition, the invasive nature of these samples does not readily permit their use in the diagnosis of *T. gondii* infection.

In conclusion, dexamethasone was used successfully to cause recrudescence of *T. gondii* infection in chronically infected mice. However blood samples were PCR positive in some instances even before immunosuppression therapy began. This would suggest that either active parasite was still circulating, or that latent cysts were occasionally breaking down releasing parasite into the blood. It would be useful to monitor the mice in such a study for a considerable time, certainly for longer than six weeks, in order to discover whether animals with parasitaemia did go on to develop toxoplasmosis. The development of a quantitative PCR may also be of value in measuring the level of parasitaemia in the host, therefore creating a system by which the risk of recrudescence could be ascertained.

CHAPTER 6

The Development of a Quantitative Polymerase Chain Reaction

6.1 INTRODUCTION

Quantitative PCR (qPCR) may be of value in assessing the relevance of positive *T. gondii* PCR results (Holliman *et al.* 1990b, Barker and Holliman 1992). Development of the qPCR has been problematic due mainly to the exponential nature of the PCR system, with small variations in amplification efficiency dramatically affecting product yield (Foley *et al.* 1993).

Several methods have been attempted for the development of qPCR systems in the study of other organisms. Pang *et al.* (1990) and Kellog *et al.* (1990) radiolabelled one of the oligonucleotides used in amplifying levels of Human Immunodeficiency Virus type 1 (HIV-1). DNA fragments were separated by gel electrophoresis, and the level of amplification in an excised band was measured using a scintillation counter. Katz *et al.* (1990) used the co-amplification of mouse "housekeeping" genes in the study of Herpes simplex virus in these animals. The mouse gene provided a control for amplification rates when the two targets were amplified. Target levels were then compared directly using gel electrophoresis, autoradiography or densitometry. Arrigo *et al.* (1990) used a similar method to quantify HIV-1. However, this system involved the use of different primers and amplification of these fragments was not completely invarient (Foley *et al.* 1993).

A target of different size was co-amplified by Wang *et al.* (1989) which was then separated by electrophoresis. The amount of DNA target was extrapolated against a standard curve while in the exponential phase. There is however potential for error with the PCR using size differences to separate the products, eg. the activity of polymerase may bias the results over different lengths of product. There is also the possibility of the development of secondary structures which may prevent primer annealing and extension.

The use of an internal control which is identical to the target apart from the existence of a restriction site may aleviate the problems encountered with other methods of qPCR (Foley *et al.* 1993). Both the target and control DNA would amplify with the same efficiency, as they would compete equally under given conditions. The ratio of products will therefore remain constant throughout amplification (Gilliland *et al.* 1990). The point

at which target and control DNA are equal can then be found by direct comparison using gel electrophoresis. This was the method chosen for development of a qPCR in this study

The P30 PCR was chosen as the bases of a qPCR because it amplifies a single copy gene, therefore limiting problems associated with multiple copy genes such as B1 which may vary in copy number. A suitable site was chosen for mutagenesis to create a novel restriction site by altering two bases. This mutated target could then be used in a single P30 PCR with the internal DS38 and DS39 primers (Chapter 2 and Figure 6.1) to produce fragments of identical length, but varying in only the two base changes. When a series of reactions is carried out containing the same sample input, but varying known amounts of mutated DNA, a competitive system will arise where the largest amount of input DNA will be amplified preferentially. Fragments may then be separated by restriction enzyme analysis, followed by electrophoresis. The point at which the amount of undigested product equals the amount of digested product (mutated DNA) identifies when input of both fragments is identical. This should reveal the original input of sample DNA.

Specific mutations can be introduced by cloning (Botstein and Shortle 1985), or by using PCR technology (Higuchi *et al.* 1988, Vallette *et al.* 1989). The "Altered sites *in vitro* mutagenesis system" (Promega) and the PCR using mismatched primers were investigated simultaneously with the aim of creating a novel restriction site in the P30 target DNA. The site chosen for mutation was at 116 (A to C) and 117 bp (G to C) to introduce a SmaI restriction site which would cut to produce fragments of 118 and 404 bp (see Figure 6.1).

The successful development of a qPCR may then be used to further examine *T. gondii* infection in human samples both as a research tool and as a means of monitoring the course of illness.

Figure 6.1 The mutation site on the P30 gene.

DS38

1 CGACAGCCGC GGTCATTCTC ACACCGACGG AGAACCACTT CACTCTCAAG 50

51 TGCCCTAAAA CAGCGCTCAC AGAGCCTCCC ACTCTTGCGT ACTCACCCAA 100

101 CAGGCAAATC TGCCC**AG**CGG GTACTACAAG TAGCTGTACA TCAAAGGCTG 150
CC

151 TAACATTGAG CTCCTTGATT CCTGAAGCAG AAGATAGCTG GTGGACGGGG 200

201 ATTCTGCTA GTCTCGACAC GGCAGGCATC AAACTCACAG TTCCAATCGA 250

251 GAAGTTCCCC GTGACAACGC AGACGTTTGT GGTCGGTTGC ATCAAGGGAG 300

301 ACGACGCACA GAGTTGTATG GTCACGGTGA CAGTACAAGC CAGAGCCTCA 350

351 TCGGTCGTCA ATAATGTCGC AAGGTGCTCC TACGGTGCAG ACAGCACTCT 400

401 TGGTCCTGTC AAGTTGTCTG CGGAAGGACC CACTACAATG ACCCTCGTGT 450

451 GCGGGAAAGA TGGGAGTCAAA GTTCCTCAAG ACAACAATCA GTACTGTTCC 500

501 GGGACGACGC TGACTGGTTG CA

DS39 (opp sense)

6.2 BACTERIAL AND MOLECULAR METHODS

6.2.1 Frequently used Solutions

see section 2.2.1.

6.2.2 Bacterial Growth Media

Bacterial growth media components were added to 1 litre final volume.

L-broth: 10 g Difco Bacto tryptone, 5 g Difco yeast extract, 5 g NaCl .

L-agar: As above plus 15 g Difco agar.

TYP broth: 16 g Difco Bacto tryptone, 16 g Difco yeast extract, 5 g NaCl, l2.5g K_2HPO_4.

Ampicillin: Media was supplemented with 50 μg ml^{-1} ampicillin (Amp) when required.

Tetracycline: Media was supplemented with 15 μg ml^{-1} tetracycline (Tet) when required.

6.2.3 Growth and Maintenance of Bacterial Cells

Escherichia coli strains were grown on L-agar or in L-broth overnight (Maniatis *et al.* 1982). Colonies on solid agar were maintained at 4°C, or alternatively long term storage was carried out at -70°C in L-broth containing 15% glycerol. Strains used in this study are described in Table 6.1.

6.2.4 Preparation of Competent *E. coli* Cells

Competent cells were prepared using the method of Chung *et al.* (1989). An overnight culture of *E. coli* was diluted 1:50 in L-broth to the early exponential growth phase in a large flask with vigorous shaking until OD_{600} reached 0.3-0.4. The cells were then harvested by centrifugation at 2 000 *g* for 10 minutes at 4°C and the resulting pellet was resuspended in 0.1 volume of transport and storage solution (TSS) (L-broth containing 10% (w/v) polyethylene glycol (PEG) 8 000, 5% (v/v) dimethyl sulfoxide (DMSO) and 25 mM $MgCl_2$ pH 6.5). Cells were then stored at -70°C for 2-3 months.

Table 6.1 *Escherichia coli* strains.

Strain	Genotype	Reference
JM109	endA1, recA1, gyrA96, thi, hsdR17 (r_k^-, m_k^+), relA1, supE44, Δ^-, Δ (lac-proAB), [F', traD36, $proA^+B^+$, $lacI^q$ ZΔM15]	Yannish-Perron *et al.* (1985)
BMH -18 mut S	thi,supE,Δ(lac-proAB),[mutS::Tn10] [F', $proA^+B^+$, $lacI^q$ZΔM15]	Kramer, Kramer and Fritz (1984)

6.2.5 Restriction Endonuclease Digestion

Restriction enzymes (Boehringer Mannheim) were used according to the manufacturer's instructions. Reactions were carried out using endonucleases at 10 units μg^{-1} with the appropriate 10X buffer supplied, and at the appropriate temperature for 2 hours. RNase was added at a final concentration of 20 $\mu g\ ml^{-1}$ when necessary.

6.2.6 Dephosphorylation of Vector DNA

Calf intestinal alkaline phosphatase (CIP) (Boehringer Mannheim) was used to remove 5'-phosphate groups from linearised vector. Reactions were carried out in 10-20 µl volumes by the addition of 10X CIP buffer (0.5 M Tris -HCl pH 9.0, 10 mM $MgCl_2$, 1mM $ZnCl_2$, 10 mM spermidine) and 0.01 units of CIP per µg of DNA used. The volume was adjusted by the addition of sterile distilled water. Incubation was carried out at 37°C for 30 minutes followed by inactivation of CIP by heating at 75°C in the presence of 5 mM EDTA. DNA was extracted with phenol / chloroform, followed by chloroform, and then precipitated by the addition of 0.1 volume 3 M sodium acetate and 2 volumes of ethanol.

6.2.7 Phosphorylation of Primers

When necessary, primers were phosphorylated at the 5' end. The reaction consisted of 100 pmol primer, 2.5 µl 10X kinase buffer (500 mM Tris-HCl pH7.5, 100 mM $MgCl_2$, 50 mM dithiothreitol (DTT), 1 mM spermidine, 10 mM ATP) and 5 units T4 polynucleotide kinase, made up to 25 µl with sterile distilled water. Incubation was carried out at 37°C for 30 minutes, followed by heating to 70°C to inactivate the kinase. Phosphorylated primers were stored at -20°C.

6.2.8 Ligation Reactions

Ligation reactions were carried out using insert and vector DNA at a ratio of 1:4. Ligation buffer (10X) (600 mM Tris-HCl, 50 mM Mg Cl_2, 10 mM DTT, 10 mM ATP, pH 7.5) and T4 ligase were supplied by Boehringer Mannheim. Reactions were carried out using between 1 and 3 units of T4 ligase in 20 µl volumes, made up with sterile distilled water. Incubation was at room temperature for 1-4 hours, or at 16°C overnight.

6.2.9 Transformation of Competent *E. coli*

Transformation of competent *E. coli* cells was carried out using the method of Chung *et al.* (1989). Ligated plasmid DNA (25-50 ng) was added to 200 µl competent cells and incubated on ice for 30 minutes. After heat shock at 37°C for 2 minutes, 0.8 ml of pre-warmed TSS was added, followed by incubation at 37°C for 1 hour with vigorous shaking. The cells were collected by centrifugation, and approximately 800 µl of supernatant was removed before the pellet was resuspended in the remaining 200 µl. Recombinants were selected by plating 50 and 100 µl aliquots on L-agar plates containing an appropriate antibiotic (see 6.2.2) and 24 µg ml^{-1} isopropyl-D-thiogalactopyranosidase (IPTG) (Bio-Rad), and 20 µg ml^{-1} X-galactose (X-gal) (Bio-Rad). After overnight incubation, recombinants appeared as white colonies against a blue background.

6.2.10 Small Scale Preparation of Plasmid DNA

Colonies were selected and inoculated into 10 ml L-broth and incubated overnight at 37°C. Cells from 1.5 ml of culture were collected by centrifugation at 9 000 g for 30 seconds, and were then resuspended in 100 µl TEG (25 mM Tris-HCl pH8.0, 10 mM EDTA, 50 mM glucose). A 200 µl volume of freshly prepared 1% (w/v) SDS and 0.2 M NaOH was added and the contents of the tube were mixed by inversion, then incubated on ice for 5 minutes. This was followed by centrifugation at 9 000 *g* for 5 minutes and removal of the supernatant containing the plasmid DNA. DNA was extracted using phenol / chloroform and then precipitated by the addition of 1 ml ethanol. The final pellet was resuspended in 50 µl sterile distilled water.

6.2.11 Large Scale Production of Plasmid DNA

This was carried out using "Magic Megapreps DNA purification system" (Promega). Cells from 600 ml of overnight culture were collected by centrifugation at 14 000 g for 20 minutes. The pellet was resuspended in "cell resuspension solution" (50 mM Tris-HCl pH7.5, 10 mM EDTA, 100 µg ml^{-1} RNase A) and then lysed by the addition of 30 ml "cell lysis solution" (0.2 M NaOH, 1% SDS). The cell suspension was neutralized with 30 ml 2.55 M potassium acetate (pH 4.8) followed by centrifugation at 14 000 g for 15

minutes. After transfer to a new bottle, a 0.6 volume of isopropanol was added to the supernatant. DNA was collected by centrifugation at 14 000 g for 15 minutes and then resuspended in 5 ml TE. Plasmid was purified by the addition of 20 ml "DNA purification resin" and passing through a "Megacolumn" using a vacuum manifold. The resin was washed with (200 mM NaCl, 20 mM Tris-HCl pH 7.5, 5 mM EDTA), diluted 1:1 with 95% ethanol, and then rinsed using 80% ethanol. DNA was eluted from the column by the addition of 3 ml sterile distilled water at 65°C followed by centrifugation at 1 300 g for 5 minutes.

6.2.12 Quantification of DNA

See 2.2.8.

6.2.13 Recovery of DNA from Agarose Gels

DNA fragments were recovered from agarose gels using methods involving the use of powdered glass as described by Vogelstein and Gillespie (1979).

Fragments Greater than 300 bp

The "Gene clean 2" kit (Bio 101) was used to recover DNA fragments larger than 300 bp from agarose gels. DNA present in agarose was excised from the gel and dissolved in 2-3 volumes of 3 M NaI at 50-55°C. A 5µl volume of silica matrix in water ("glassmilk") was then added and the mixture was vortexed then left at room temperature for 5 minutes. The DNA-"glassmilk" matrix was centrifuged at 9 000 *g* for 5 seconds and washed three times with "NEW wash" (a solution of NaCl, Tris, EDTA and ethanol). DNA was then eluted in 5 µl sterile distilled water at 50°C for 3 minutes, and the "glassmilk" removed by centrifugation at 9 000 *g* for 30 seconds.

Fragments Smaller than 300 bp

Small fragments of DNA were recovered from agarose gels using the "Mermaid" kit (Bio 101) following manufacturer's instructions. The desired DNA band was cut from the gel and added to 3 volumes of "high salt binding solution". After the addition of 8 µl "glassfog" (a silica matrix) per µg DNA, the tube was vortexed for 10 minutes followed

by centrifugation at 9 000 *g* for 5 seconds. The supernatant was removed and the pellet washed with 300 µl "ethanol wash". After centrifugation and two further washes, the DNA was eluted from the "glassfog" by resuspending the pellet in 10 µl sterile distilled water, and then incubating at 55°C for 5 minutes. This was followed by centrifugation at 9 000 *g* for 1 minute, after which the supernatant was transferred to another tube.

6.2.14 Di-deoxy Chain Termination Sequencing

DNA sequencing was carried out using the method of Sanger *et al.* (1977). All reagents were supplied in the T7 sequencing kit (Pharmacia), except [^{35}S]dATP (Amersham). DNA template (2 µg) was denatured by the addition of 0.4 M NaOH and incubating at room temperature for 10 minutes. This was followed by the addition of 0.3 volume of 3 M sodium acetate (pH 4.8), 0.7 volume of distilled water and 6 volumes of ethanol to precipitate DNA. After centrifugation at 9 000 g for 10 minutes, the pellet was washed with 70% ethanol and then air dried and redissolved in 10 µl distilled water

Annealing reactions were carried out by the addition of 2 µl of "annealing buffer" (solution containing $MgCl_2$ and DTT) and 2 µl of primer (final concentration 0.8 µM) to 10 µl (2 µg) denatured template and incubating at 37°C for 20 minutes.

T7 polymerase was diluted in "enzyme dilution buffer" (containing glycerol, BSA and DTT) to result in 3 units in 2 µl for each reaction. An enzyme premix was made by adding the diluted enzyme to 1 µl (10 µCi) of [^{35}S]dATP, 3 µl "labelling mix A" (dCTP, dGTP and dTTP). Labelling reactions were then carried out by adding the premix to the annealed template and primer, and incubating at room temperature for 5 minutes to allow incorporation of [^{35}S]dATP. The reactions were terminated by the addition of 4.5 µl pre-warmed 'A', 'C', 'G' and 'T' mixes, which contained deoxy and di-deoxy forms (d/ddNTP's) of the respective bases. After incubation at 37°C for 5 minutes, 5 µl "stop solution" was added and samples were stored at -20°C.

Samples were then resolved using 6% PAGE which was prepared using 40% acrylamide stock (38% (w/v) acrylamide, 2% (w/v) bis-acrylamide), urea at a final concentration of

7 M, and 10X TBE (1 M Tris-HCl, 0.86 M boric acid, 20 mM EDTA). Prior to pouring the gel, 0.1% (w/v) APS and 0.05% (v/v) TEMED were added. Electrophoresis was carried out using 1X TBE and S2 (BRL) apparatus at 50 watts constant power. Gels were pre-run for 30 minutes before adding 3.5 µl sample, which was freshly denatured at 80°C for 2 minutes. Further samples were added 2 hours later, and electrophoresis continued for a further 3-4 hours. Gels were fixed using 10% (v/v) methanol, 10% (v/v) ethanoic acid and then transferred onto Whatman 3MM filter paper and dried under vacuum at 80°C for 30 minutes.

Dried gels were exposed to X-ray film (Fuji) in radiography cassettes to detect [^{35}S]-labelled products. Film was developed in "Photosol" CDL8 developer and fixed in "Photosol" CF40 fix (Amersham).

6.2.15 DNA Sequencing using the fmol System

The "fmol sequencing system" (Promega) utilizes PCR technology in di-deoxy termination reactions. Extension / termination reactions were carried out by mixing 100 ng template DNA, 0.8 µM primer, [^{35}S]dATP (10 µCi µl^{-1}) and "sequencing buffer" with the addition of sterile distilled water to a final volume of 16 µl. Sequencing grade *Taq* DNA polymerase (5 units) was then added. A 4 µl volume of this mixture was added to each of four tubes containing 2 µl d/ddNTP's ('A', 'C', 'G' and 'T'). After mixing, the reactions were overlayed with 20 µl mineral oil and placed in a thermal cycler (Hybaid) at 95°C. Thirty cycles were carried out (denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 70°C). Reactions were stopped by the addition of "stop solution" and were then processed using PAGE as described in 6.2.13.

6.3 ATTEMPTED MUTAGENESIS USING THE ALTERED SITES SYSTEM

The "Altered Sites *in vitro* Mutagenesis System" (Promega) was used in an attempt to introduce two base pair changes necessary to create a novel restriction endonuclease site. This system consists of a mutagenesis vector (pALTER-1) which contains two genes for antibiotic resistance, and a procedure for selection of oligonucleotide-directed mutants. The Tet gene is always functional, whereas the Amp gene is inactivated. After cloning

into pALTER, "helper phage" is used to make the plasmid single stranded. A primer provided with the kit, which restores Amp resistance is annealed to the single stranded DNA (ssDNA) along with the mutagenic primer complementary to the site, which contains the two base changes. Subsequent synthesis and ligation of the mutant strand links the two primers, and the DNA may then be used to transform BMH 71 cells. A second round of transformation in JM109 can then be carried out to separate mutant plasmids.

6.3.1 The pALTER-1 Vector

The pALTER-1 vector is a phagemid containing the origin of replication from a single stranded DNA (ssDNA) bacteriophage (see Figure 6.2). The multiple cloning site (MCS) is flanked by SP6 and T7 RNA polymerase promoters and is inserted in the lac-Z open reading frame (ORF) which encodes ß-galactosidase. The MCS allows cloning in a variety of sites using different restriction endonucleases which disrupts the lac-Z reading frame and so ß-galactosidase is not produced. The presence of recombinant colonies can then be detected by growing the cells on an agar plate containing X-gal, which cannot be utilized and so these colonies appear white against a background of blue colonies.

6.3.2 Cloning into the pALTER-1 Vector

P30 PCR product (522 bp) was recovered from an agarose gel using "Gene clean 2" and was then used in a ligation reaction with pALTER which had been digested with Sma I. This enzyme digests DNA to produce blunt ends and so was chosen because the PCR products should theoretically be blunt ended, therefore ligation should be possible. The ligated plasmid was transformed into competent JM109 cells, and incubated on L-agar containing X-gal , IPTG and Tet. This did not result in any recombinant colonies, and so the TA PCR vector (Invitrogen) was used in an attempt to clone the P30 fragment. This vector which contains an overhanging 'T' is recommended for cloning PCR products, which often contain an extra 'A' at the end of the fragment. Once cloned, the vector may be digested with EcoR I and then sub-cloned into pALTER for further investigation. However, transformation was again unsuccessful, with no recombinants growing on L-agar with L-Amp.

Figure 6.2 pALTER-1 vector

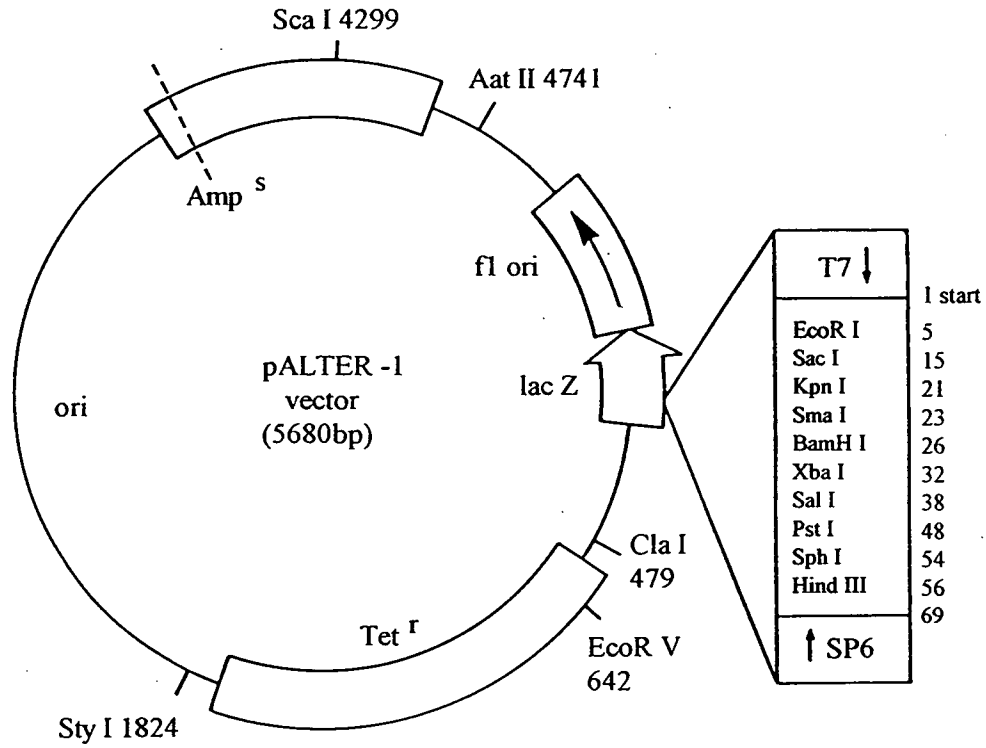


Figure 6.3 Primers used to clone P30 PCR products

DS38+	ATA **GAA TTC** CGA CAG CCG CGG TCA TTC TC
DS39+	CCT GCT GCG ACT GAC CAA CGC **TTA AGT** TT

Bold regions show EcoR I sites.

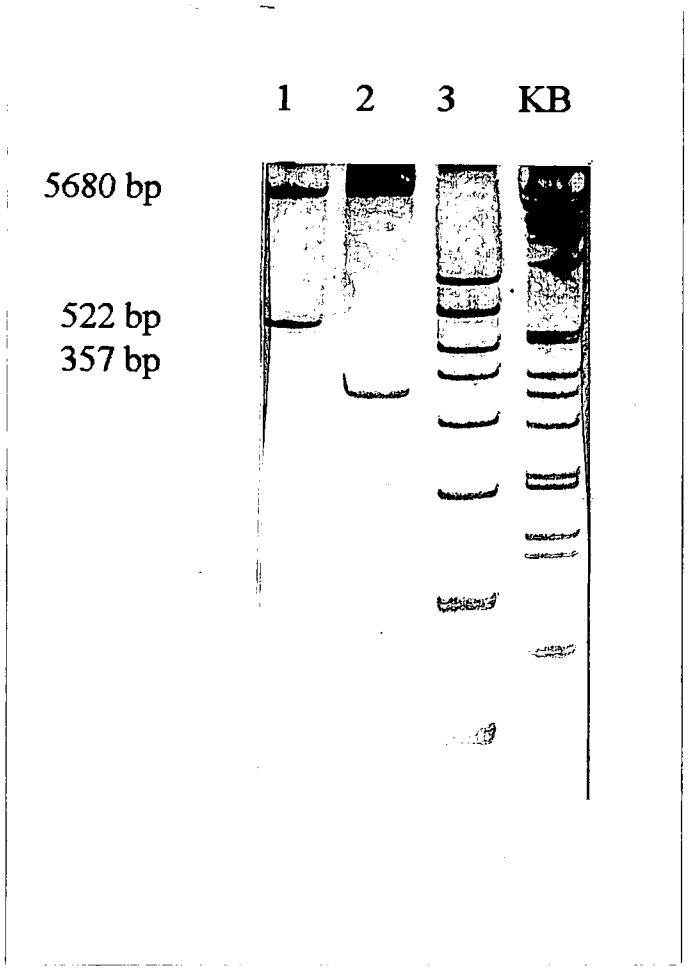
New primers (DS38+ and DS39+) which included EcoR I ends (see Figure 6.3) were then used in PCR reactions with original P30 product. The resulting PCR products (540 bp) and pALTER were digested with EcoR 1 and then ligated. Transformation into JM109 cells produced 20 recombinants, and small scale plasmid preparations followed by EcoR I digestion obtained a product of 532 bp.

Digestion of the recombinant plasmid was carried out using Sac I to find the orientation of the fragment. There is one Sac I site in the MCS, and one in the PCR fragment 165 bp into the 522 bp fragment. Two fragments of 5680 bp and 357 bp were produced (see Figure 6.4). The orientation of the fragment is shown in Figure 6.5.

6.3.3 Preparation of Phagemid ssDNA

An overnight culture was used to inoculate TYP broth containing 15 μg ml^{-1} Tet. This was incubated at 37°C for 30 minutes with vigorous shaking before infection with 10 helper phage particles per cell ("helper" phage R408), and further incubation at 37°C overnight. Cells were separated by centrifugation at 12 000 *g* for 15 minutes, and helper phage was precipitated from the supernatant by the addition of 0.25 volume 3.75 ammonium acetate (pH 7.5) and 20% PEG 8000 on ice. After centrifugation at 12 000 g for 15 minutes, the pellet containing phagemid DNA was resuspended in 400 μl TE. An equal volume of chloroform was added to lyse the phagemid DNA. After centrifugation at 12 000 *g* to remove excess PEG, DNA was extracted from the supernatant using phenol / chloroform followed by ethanol precipitation at -20°C. DNA was collected by centrifugation at 12 000 *g* and the pellet was washed with 70% ethanol, air dried and resuspended in 20 μl sterile distilled water. Single stranded DNA was run on 0.8% agarose to reveal a band at 1.5 Kb (data not shown). The expected running point of the DNA was 6.2 Kb, however ssDNA often runs much lower than dsDNA, making size estimation difficult.

Figure 6.4 Sac I digestion of pALTER plus P30 fragment



Lane 1: EcoR I digest of the recombinant plasmid

Lane 2: Sac I digest of the recombinant plasmid

Lane 3: DNA ladder (Cambio)

Figure 6.5 Orientation of the cloned P30 fragment.

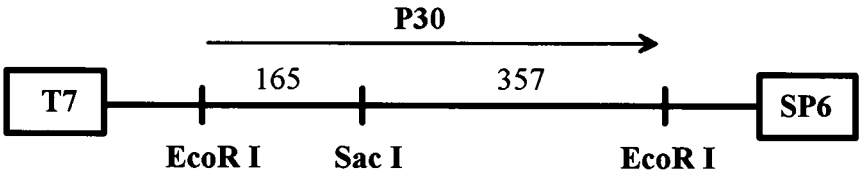


Figure 6.6 The mutagenic primers.

a) P1 AGG CAA ATC TGC CCC CCG GGT ACT ACA AGT

b) P2 ACT TGT AGT ACC CGG GGG GCA GAT TTG CCT

6.3.4 Mutagenesis Procedure

Mutagenesis involved the annealing of the Amp repair oligo-nucleotide described earlier (see 6.3), and the mutagenic primer to the ssDNA template. The mutant strand was then synthesised using T4 DNA polymerase. The reaction consisted of 0.05 pmol recombinant pALTER ssDNA, 0.25 pmol Amp repair oligonucleotide (phosphorylated), 1.25 pmol mutagenic primer (phosphorylated) (Figure 6.6a), 2 µl 10X annealing buffer (200 mM Tris-HCl pH7.5, 100 mM MgCl2, 500 mM NaCl) and distilled water to a volume of 20 µl. After heating at 70°C for 5 minutes, the reaction was cooled slowly to room temperature to allow annealing. This was followed by adding (on ice) 3 µl "synthesis 10X buffer" (100 mM Tris-HCl pH7.5, 5 mM dNTP's, 10 mM ATP, 20 mM DTT), 1 µl (10 units) T4 DNA polymerase, 1 µl (2 units) T4 DNA ligase and 5 µl sterile distilled water. Incubation was carried out at 37°C for 90 minutes to allow mutant strand synthesis and ligation.

The DNA was then used in a transformation with BMH71-18 mut S (see Table 6.1). Mutants were selected by overnight culture in the presence of Amp.

6.3.5 Results using the pALTER System

Many white, and several blue colonies were present after overnight selection. Small scale plasmid preparations and Sma I digestion was carried out on twenty white colonies. Reaction products were then analysed by gel electrophoresis which revealed that they all contained the P30 insert, but none contained the Sma I site. The pALTER system was repeated, but was again unsuccessful in creating the mutated site.

6.4 MUTAGENISIS USING PCR TECHNIQUES

A method based on that of Higuchi *et al.* (1988) and Vallette *et al.* (1989) was used instead of bacterial cloning methods to produce a DNA fragment containing a mutation site. The system uses primer directed mutagenesis to produce a deletion, insert or substitution at any point of the fragment. By using the mismatched primer used in 6.6a (P1), and another containing the same changes at the same site on the opposite strand

(P2), a Sma I restriction site may be introduced (see Figure 6.6b). This method is shown in Figure 6.7.

6.4.1 Reactions Using Mismatched Primers

Primers P1 and P2 were used in separate reactions with primers DS39 and DS38 respectively. Thirty cycles were carried out using P30 PCR reaction conditions, with the exception of the annealing temperature which was reduced to 50°C. This produced fragments of 131 bp (DS38 and P2) and 421 bp (DS39 and P1) (Figure 6.8).

6.4.2 Combination of the PCR Products

PCR products were run on 1% agarose and the fragments were recovered by "Geneclean" and "Mermaid". DNA from both these fragments was denatured and renatured and then added to one PCR reaction using external primers only (DS38 and DS39) which produced a 522 bp fragment. Fragments of 118 bp and 404 bp were obtained respectively when these products were digested with Sma I (Figure 6.9).

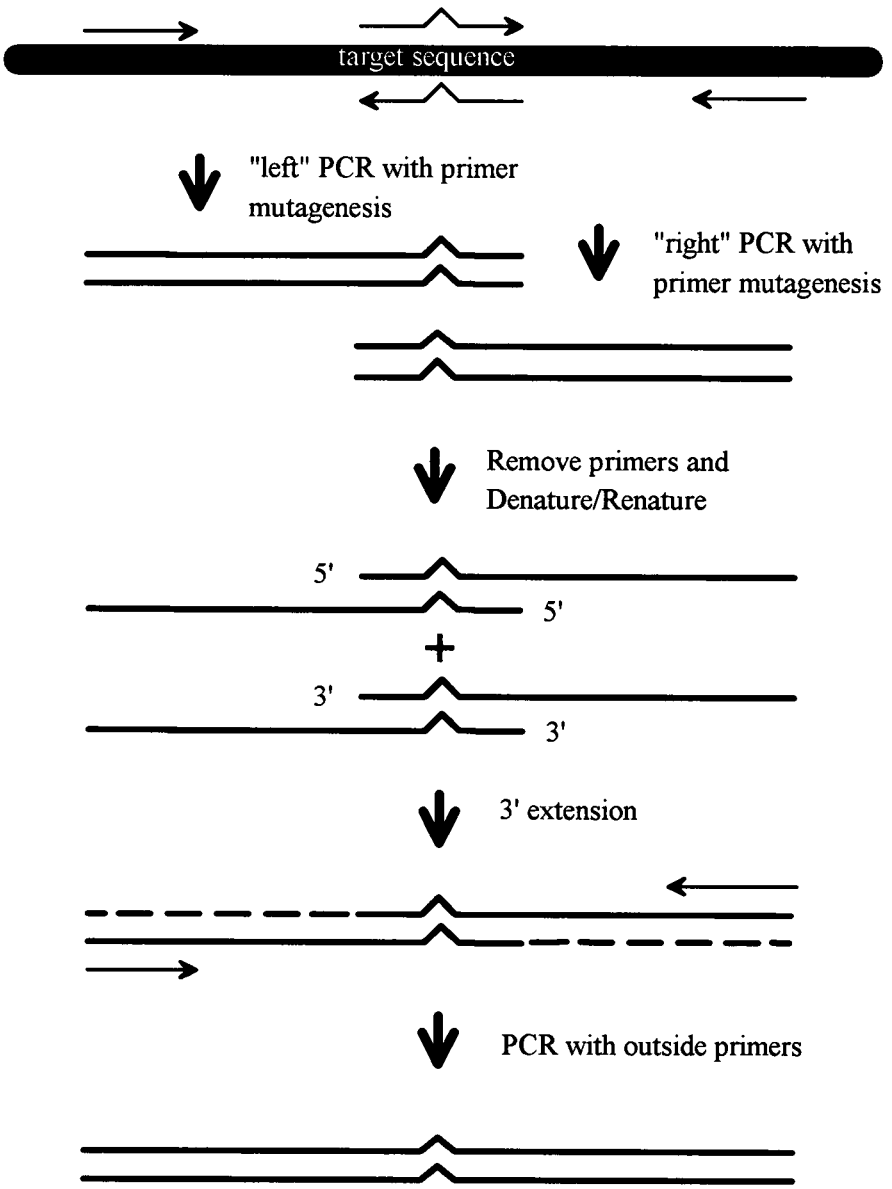
6.4.3 Cloning the Mutated Fragment

Although qPCR could be carried out using the mutated fragment as a control in samples, it would be advantagous to consider the effects of other DNA from the host present in the system. Mutated P30 DNA was cloned into a vector for further manipulation. PCR was repeated using DS38+ and DS39+ with the mutated 522 bp fragment to introduce EcoR I sites for cloning into pBS SK+, and this was used to transform JM109 cells. Cloned fragment could be used to create any amount of mutated template needed for the qPCR.

6.4.4 Sequence Analysis

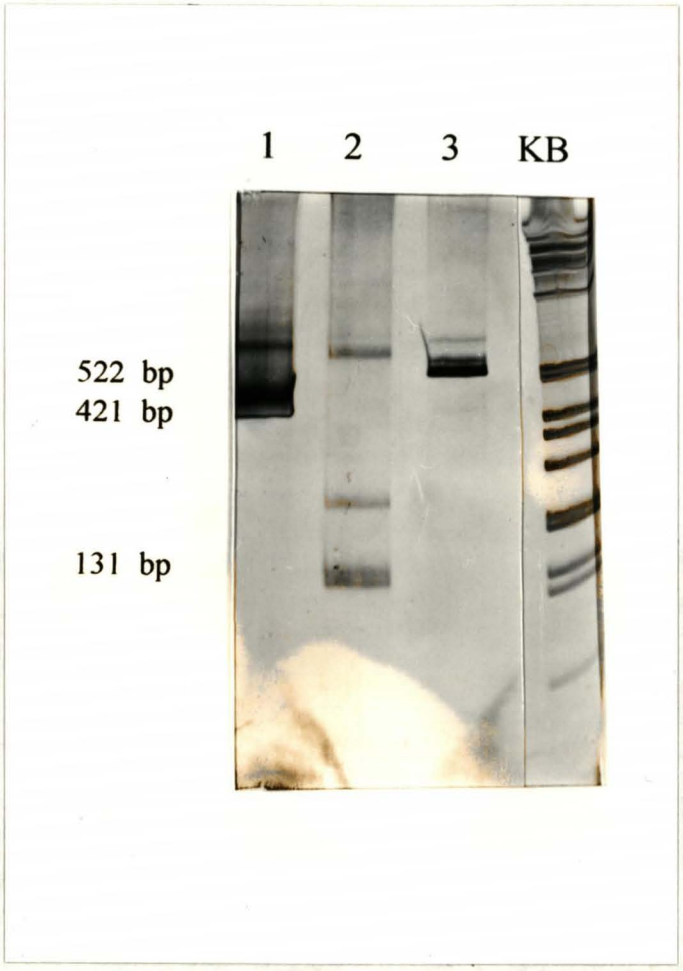
T7 sequence analysis (Pharmacia) (6.2.14) was unsuccessful in the analysis of the mutated DNA product. However the fmol (Promega) (6.2.15) system revealed base changes of 'A' to 'C' and 'G' to 'C' at bases 116 and 117 bases respectively. This confirmed that *in-situ* PCR mutagenesis was successful.

Figure 6.7 Mutagenesis using the polymerase chain reaction.



(Adapted from Higuchi *et al.* 1988)

Figure 6.8 The PCR using mismatched primers

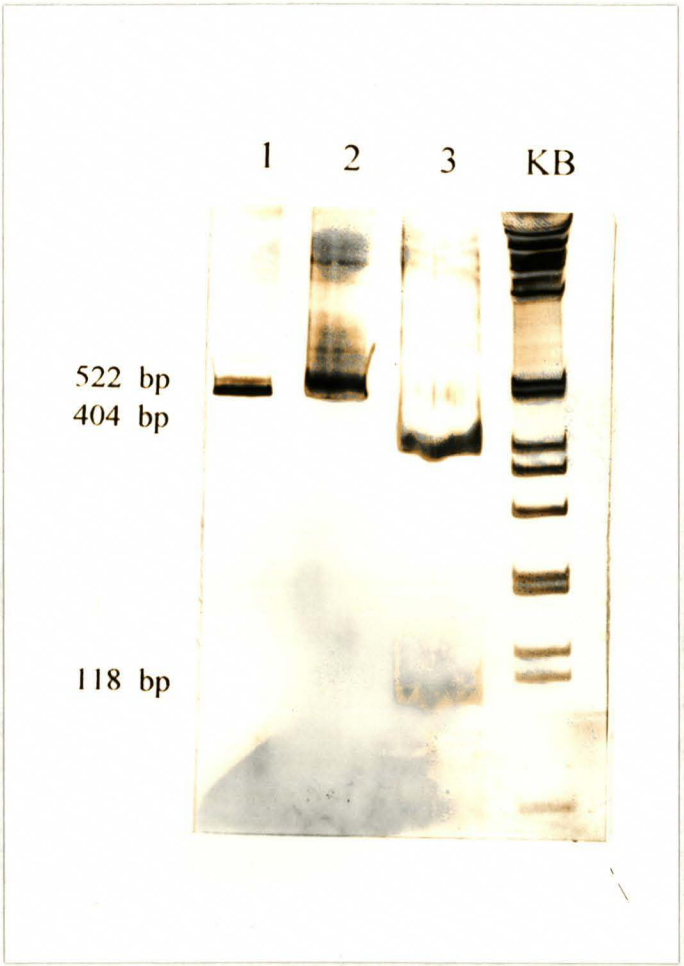


Lane 1: DS39 + P1

Lane 2: DS38 + P2

Lane 3: DS38 + DS39

Figure 6.9 Sma I digestion of the PCR products using mismatched primers



Lane 1: DS38 + DS39

Lane 2: DS38 + DS39 (using the products from the mismatched PCR)

Lane 3: Sma I digest of products in Lane 2

6.5 QUANTITATIVE PCR

Control reactions were carried out to investigate the accuracy of qPCR using the mutated P30 fragment in a competitive reaction. A series of titrations was carried out using two amounts of *T. gondii* DNA (1 pg and 10 pg) with varying amounts of mutated DNA in pBS SK+ (1 pg, 0.5 pg, 0.1 pg, 10 fg, 1 fg, 0.5 fg, 0.2 fg, 0.1 fg). Forty cycles were carried out under normal P30 PCR conditions (2.2.10).

There is approximately one copy of P30 fragment in 0.07 pg of *T. gondii* DNA ($7x10^7$ bp). However there are $2x10^4$ copies in the same amount of vector plus mutated DNA ($3.5x10^3$ bp). As 1 pg of *T. gondii* DNA was added to the reactions, this equalled approximately 14 P30 copies or parasites. An equivelent number of copies of mutated P30 in pBS SK+ would be present in 0.49 fg. Equilibrium was reached between lanes 6 and 7 (Figure 6.10) which contained 0.5 fg and 0.2 fg of mutated fragment respectively. When 10 pg of *T. gondii* was used in a qPCR, band intensity was roughly equal when between 2 and 5 fg of mutated fragment plus vector was added to the reaction (data not shown). The results were visible by eye and the results were relatively simple to interpret, however the smaller 118 bp fragment must also be considered and so densitometry was also applied to the gel (Figure 6.11). In the qPCR using 1 pg of *Toxoplasma* DNA this revealed that the point at which the levels of DNA were equal did lie between 0.5 fg (the digested bands were aproximately twice as intense), and 0.2 fg (the digested bands were aproximately one third as intense) ie. between lanes six and seven.

6.5.1 Quantitative PCR with Patient Samples

PM tissue samples which were previously recorded as PCR positive by either B1 or P30 PCR (4.3.2) were analysed using qPCR. A total of 26 tissue samples from seven patients which had been treated earlier (see 4.2.3) and stored at -70°C were tested. As before, 5 µl of a 50 µl preparation from 0.2 g of tissue was added to each PCR along with known dilutions of mutated DNA (2 pg, 0.5 pg, 0.1 pg, 10 fg, 5 fg, 1 fg, 0.1 fg).

Figure 6.10 Quantitative PCR products after Sma I digestion to reveal true products (522 bp) and mutated DNA products (404 bp and 118 bp)

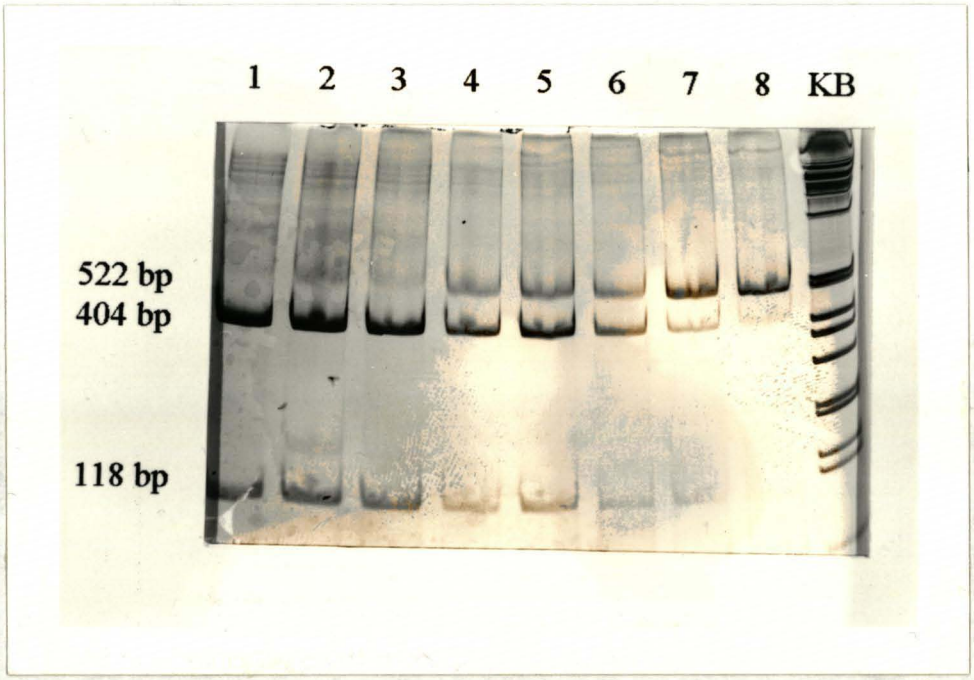
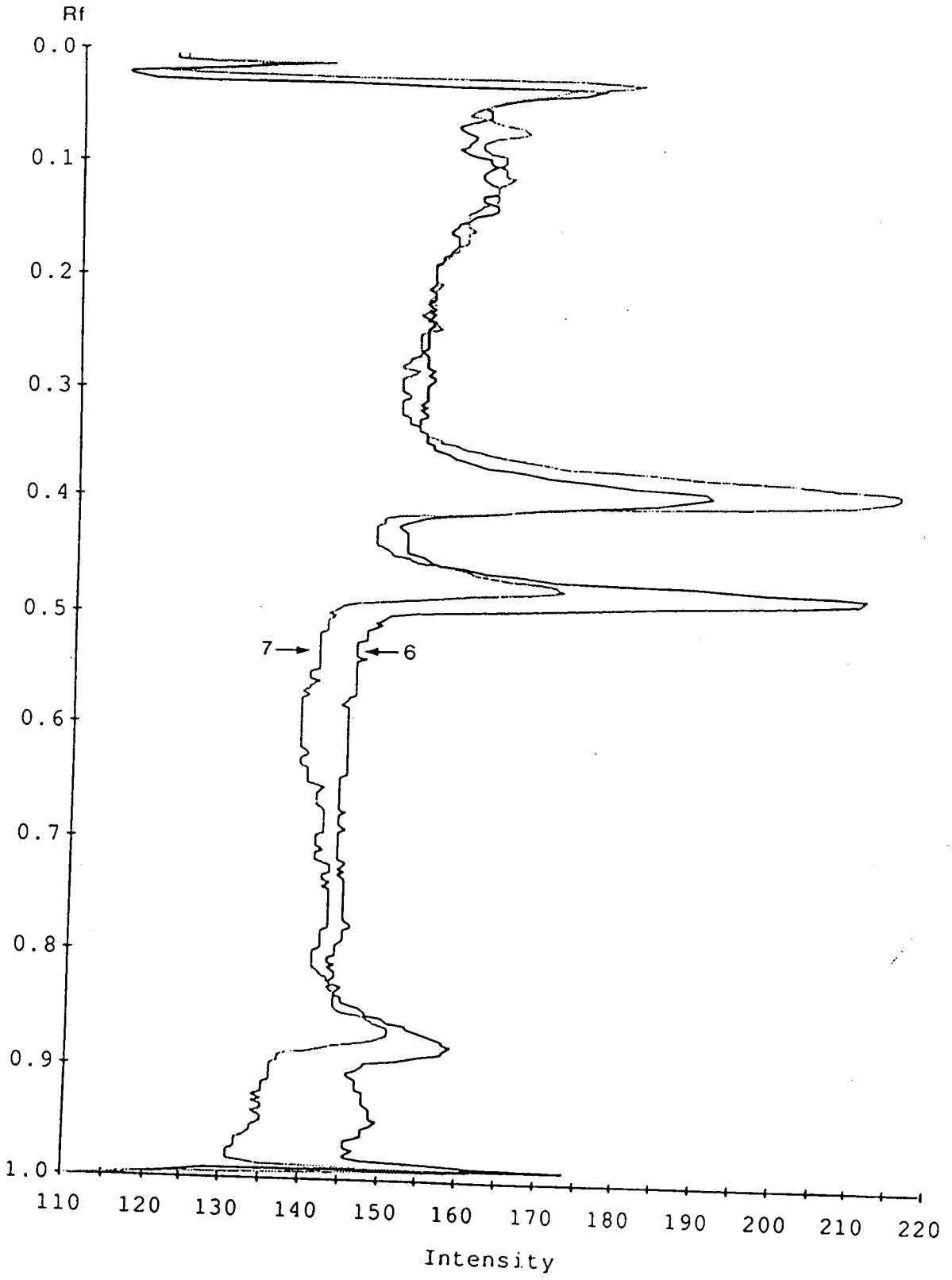


Figure 6.11 Densitometry of the qPCR products (lanes 6 and 7) from gel (Figure 6.10)



Toxoplasma DNA was quantified in 26 samples of DNA from these patients. However, only one set of titrations of mutated DNA from 2 pg to 0.1 fg was used due to the limited amounts of prepared samples available. Testing using the qPCR followed by Sma I digestion and gel electrophoresis enabled crude quantification of DNA from 56 000 to 2.8 P30 copies in one tenth of a 0.2 g tissue preparation.

Tissue samples from three patients who died from active toxoplasmosis (one from group 3 and another two who died before the study commenced) contained 100 to 500 times as many copies of the P30 fragment than those who were IgG positive with no clinical symptoms at death (see Table 6.2). Samples from the CNS and myocardium generally contained a higher number of parasites in comparison to other tissue types.

6.5 DISCUSSION

The lack of success using the pALTER Mutagenesis System was probably due to the failure of helper phage to successfully make ssDNA. The presence of dsDNA in the reaction would cause problems with primer annealing to the template. However, the Amp resistance oligonucleotide did result in the production of many Amp (mainly white), resistant colonies. Therefore there may also have been a problem with the mutagenic primer which meant that it was not incorporated in the synthesis of DNA.

PCR technology was successful in producing a novel restriction site in the P30 gene. The P30 qPCR system was shown to be accurate in the assessment of parasite DNA in tests involving known amounts of *T. gondii* DNA (6.4.4).

Quantification of DNA in human samples was possible, but only at crude levels due to the lack of prepared sample volumes. If larger sample volumes had been prepared, it would have been possible to carry out finer titrations close to the level detected by an initial series of reactions, and so therefore obtain a more accurate assessment of parasite numbers. The need for large volumes of prepared sample for full assessment would not normally be problematic in tissue testing. However samples such as blood and CSF may be in insufficient quantity to allow accurate quantification of parasite DNA.

Table 6.2 The quantitative PCR using human tissue samples.

Patient	Sample	B1 PCR	P30 PCR	qPCR (Copies in 1/10 of 0.2 g tissue)	Status at Death
1	Brain	+	+	1420	Clinical toxoplasmosis
	Thymus	+	-	142	
2	Spinal Cord	+	-	< 2.8	IgG +
3	Brain	-	+	28	IgG +
	Spinal Cord	-	+	14	
	Lymph Node	-	+	2.8	
	Thymus	-	+	2.8	
	Myocardium	-	+	142	
4	Myocardium	+	+	2850	Clinical toxoplasmosis
5	Brain	+	+	285	Clinical toxoplasmosis
	Spinal Cord	+	+	142	
	Thymus	+	+	285	
	Lymph Node	+	+	285	
	Myocardium	+	+	285	
	Bone Marrow	+	+	285	
	Adrenal	+	-	<2.8	
	Spleen	+	-	<2.8	
	Pancreas	+	-	<2.8	
	Lung	+	-	<2.8	
	Liver	+	-	<2.8	
	Rectal Muscle	+	+	142	
	Colon	+	-	<2.8	
6	Brain	+	-	<2.8	IgG +
	Spinal Cord	+	-	<2.8	
7	Brain	+	-	28	IgG +
	Spinal Cord	+	-	142	

Large amounts of DNA were located in the CNS and myocardium of patients, which is compatible with the areas problematic in *T. gondii* recrudescence due to AIDS and transplant therapy (Luft *et al.* 1983a, Speirs *et al.* 1988, Grant *et al.* 1990, Luft and Hafner 1990, Dannemann *et al.* 1991). The detection of DNA in tissues of *Toxoplasma* IgG positive individuals with no signs of infection was probably due to the existence of tissue cysts. Increased levels of parasite in tissue may signify active infection rather than bradyzoites in tissue cysts. This may be of value as a research tool, but is of limited value in diagnosis, especially if invasive techniques such as biopsy are to be used. The system may be of greater value in examination of blood samples, where parasite is not usually detected except in cases of acute infection (van de Ven *et al.* 1991, Dupouy-Camet *et al.* 1993). It would be interesting to test samples such as blood and urine from *T. gondii* IgG positive individuals who were PCR positive but who do not appear to have clinical illness (4.3.2) using qPCR. This may reveal whether detected parasite was due to intermittent cyst breakdown (where DNA is detected at low levels), or to a mild parasitaemia which may be problematic in the future (where DNA is detected at higher levels). The qPCR would also be of value in following the effects of drug therapy using blood samples from patients. However serial blood sampling would be required using larger samples or preparations diluted in larger volumes of buffer (200 µl compared to 50 µl). This may in turn result in a dilution effect, where samples containing low levels of parasite are negative using the PCR.

CHAPTER 7

Discussion

7.1 INTRODUCTION

The majority of methods currently used to diagnose *T. gondii* infection involve the detection of IgG and IgM in blood samples. Direct methods such as microscopy and culture are insensitive or time consuming and so are of little diagnostic value. In the 1970's and 1980's work concentrated on the development of an antigen ELISA as a sensitive and efficient way of detecting the parasite directly. The need for a such a test became more obvious as the incidence of AIDS increased in the population, and serological testing failed to detect recrudescence of infection in these and other immunocompromised patients. Because of a lack of specific tests invasive procedures such as brain biopsy were often used to confirm the diagnosis following MRI and CAT scans. Previous studies revealed success rates varying from 5.7% (van Knapen and Panggabean 1977) to 63.6% (Araujo and Remington 1980) using the antigen ELISA. More recently the PCR has been used to detect a wide variety of pathogens from human and animal samples and initial work on the detection of *T. gondii* using this technique investigated the use of the P30 gene (Savva *et al.* 1990), and the B1 gene (Burg *et al.* 1989). Both PCR systems were shown to detect parasite in mock samples (Joss *et al.* 1993, Savva *et al.* 1990), and also in limited clinical samples (Holliman *et al.* 1991a, 1991b, Verhofstede *et al.* 1993).

The primary aim of this study was to develop and assess a sensitive and specific system to detect *T. gondii*. This could then be used to diagnose toxoplasmosis in immunocompromised patients and follow the effects of drug therapy, as well as investigate the complex relationship between the parasite and the host.

7.2 THE DETECTION OF *T. GONDII*

In the present study both the antigen ELISA and the PCR were successful in detecting *T. gondii*, however the PCR was found to be approximately 500 times more sensitive than the ELISA. Other workers have developed the PCR to detect a wide variety of organisms in which conventional serological screening has been problematic, such as HIV (Laure *et al.* 1988), Papillomavirus (Morris *et al.* 1988), CMV (Shibata *et al.* 1988, Gozlan *et al.* 1992) and *Pneumocystis carinii* (Wakefield *et al.* 1990). The technique has

greatly increased sensitivity in comparison to other tests and has been widely introduced in diagnostic and research laboratories. If chosen carefully, primers result in the amplification of specific products with great sensitivity, however consideration must be given to the specialised facilities and equipment required for this technique which may not be currently available in many laboratories. These technical problems may therefore limit its use as a diagnostic tool.

Further work concentrating on the PCR included experiments under controlled conditions to investigate its use in a diagnostic setting, and to study parasite / host interaction. Both the B1 and the P30 PCR were used successfully to detect DNA from between one and five parasites in a sample. However the B1 system was found to be more sensitive, enabling the detection of DNA from one parasite by gel electrophoresis. This sensitivity is due to the repetitive nature of the B1 gene, in comparison to P30 which is a single copy gene (Verhofstede *et al.* 1993). Initial work suggested that this great sensitivity may cause contamination problems; an area of concern in the diagnostic setting. Precautions were therefore taken to avoid contamination which included the use of positive displacement pipettes, plugged tips, the use of gloves, and U.V. irradiation of equipment. The negative controls contained in every run ensured that false positive results were avoided, and it was demonstrated that PCR may be used in the routine diagnosis of *T. gondii* infection.

Both the B1 and P30 PCR detected a "window" of parasitaemia in experimentally infected sheep (Chapter 3). Parasite was detected in samples of lymph and blood several days after infection and then for three to five days before disappearing. Therefore a primary infection involves a parasitaemia which may allow the parasite to invade and encyst in many tissues. When immunocompetent patients with primary toxoplasmosis were observed and followed, parasite was found to persist in the blood for a longer period than that observed in sheep. This was most likely due to the use of the incomplete strain (S48) of *T. gondii* used to inoculate sheep, compared to wild type infections in the patients.

Parasitaemia was found to be transient in immunocompromised patients suffering from recrudescence of a chronic infection, however parasite was detected in blood samples from patients up to one month prior to any clinical evidence of infection. The detection "window" lasted for only a short time, often for only a few days, indicating that sample timing was therefore critical in the detection of parasitaemia. This result also reveals that recrudescence is not completely due to localised reactivation of tissue cysts, but may be due to a more widespread parasitaemia as suggested by Luft and Remington (1992).

T. gondii DNA was detected in the blood of several patients with no clinical evidence of infection. These patients had a slightly higher CD4+ cell count than those with clinical toxoplasmosis, therefore DNA may be detected from intermittent cyst breakdown or from the occasional breakdown and release of tachyzoites. This may still be controlled by the hosts limited immunological response, but could indicate that the patient is at risk in the future if their CD4+ cell count drops further, allowing a greater and more widespread parasitaemia.

Parasite was not detected in blood samples from one patient with confirmed toxoplasma encephalitis, but tissue samples obtained at PM examination were found to contain *T. gondii* DNA using the PCR. There is therefore a question as to whether the parasitaemia was missed, or whether localised reactivation had occurred. Localised reactivation seems unlikely in this case because of the wide range of tissues involved.

7.3 THE ANIMAL MODEL OF RECRUDESCENCE

The mouse model of recrudescence of *T. gondii* infection was developed to clarify several points regarding parasite / host interaction. Firstly, only one third of patients with AIDS and chronic *T. gondii* infection suffer any clinical signs of recrudescence (Grant *et al.* 1990), therefore there is a need to identify those at risk. Secondly, the incidence of parasitaemia required further investigation into the level and timespan over which the parasite may be detected in relation to clinical evidence of toxoplasmosis.

Dexamethasone treatment resulted in increased mortality in chronically infected mice. However two other effects were also observed: an increase in the number of mice with evidence of parasitaemia, and an increase in pathological evidence of active infection. The rate of recrudescence was similar to that in the AIDS population with past exposure to the parasite, with both having a occurence of 30-35%. This similarity was not seen in previous models developed to investigate recrudescence where nearly all mice died as a result of immunosuppressive therapy (Lindberg and Frenkel 1977, Johnson L 1992, Vollmer *et al.* 1987). In both the mouse and the human population not all individuals with *T. gondii* DNA in the blood showed clinical signs of illness. Examination of samples obtained at PM examination revealed a high number of brain samples from chronically infected animals to contain parasite, and an increase in the number of positive heart samples from sick animals. Blood samples were found to be of limited diagnostic value because of the number of samples in which DNA was detected, but with no clinical symptoms of toxoplasmosis within the timescale of the study. Therefore it would be worth pursuing this model over a longer time scale to see if animals with parasitaemia do eventually become ill. A similar effect, where parasite was detected in patients without clinical signs of infection was recorded by Hassl *et al.* (1988) using an antigen ELISA. They concluded that the presence of antigen was not indicative of an active infection, contradicting van Knapen and Panggabean (1977) and Araujo and Remington (1980) who recognised the importance of circulating parasite. This study highlights the presence of a parasitaemia prior to clinical evidence of recrudescence, however the detection of parasite in the blood did not necessarily mean that toxoplasma encephalitis would occur within the six week period of this experiment.

7.4 QUANTITATIVE PCR

The qPCR was successfully used to assess the level of infection in human samples at PM examination. In these samples, levels of parasite were higher in those patients who had clinical evidence of toxoplasmosis, compared to those with a chronic infection. However it was not possible to differentiate between active and latent infection, and further investigation would be required to establish a cut-off point between the two stages of infection.

The cloned mutant DNA created for the qPCR would also be useful as a control in all samples tested using the PCR. It could be amplified with the sample directly and negate the need for testing samples containing a known amount of DNA in a second reaction to eliminate false negative results (Chapter 4).

7.5 FUTURE WORK

As well as investigating recrudescence in the immunocompromised host, the PCR could also be used to assess the risks of damage to the foetus when the mother contracts a primary *T. gondii* infection during pregnancy. An animal model, using mice for example, may reveal the level of damage associated with different levels of parasite in the amniotic fluid. Quantitative PCR will therefore be useful in such an investigation, with the ability to differentiate between low and high levels of parasite.

Dexamethasone may also be of value in the investigation of other infections which affect the immunocompromised host. A similar model has been used successfully to study *Pneumocystis carinii* (Powles *et al.* 1992,) and the problems associated with CMV, Herpes simplex (Katz *et al.* 1990) and *Mycobacteria* (Saboor *et al.* 1992) in AIDS patients may be studied using this method. As these organisms result in chronic infection and can cause symptoms similar to those seen in toxoplasmosis in the CNS, it may be possible to improve diagnosis by using one sample to test for a range of these pathogens.

The model may also be used to study genetic differences in the control of recrudescence by investigating different strains of mice (Suzuki *et al.* 1991, McLeod *et al.* 1989). This may in turn lead towards a method of investigating genetic differences in animals prone to recrudescence, and therefore highlight those at risk.

The qPCR could be applied to the mouse model to find if only high levels of parasitaemia result in recrudescence, therefore revealing vulnerable individuals. This may then be extrapolated to patient samples, and be used to monitor the affects of therapy.

Although the qPCR detected different levels of parasite in various human tissues, it was not used to differentiate between active and latent infection in this study. It would be possible to further develop the system to produce a cut-off point above which the levels of DNA indicate active parasite, but a more accurate method of locating the active tachyzoite would be the amplification of RNA. This technique has been used by others to detect latent Herpes simplex (Katz *et al.* 1990) and HIV (Arrigo *et al.* 1990, Pang *et al.* 1990). However this would be complex to develop as the process would involve reverse transcription to make cDNA from the RNA present, followed by amplification (RT PCR). There would also be a risk that genomic DNA coding for the same product may be amplified (Gilliland *et al.* 1990). It would therefore be necessary to span introns and exons in the amplified region which would vary the size of amplification product, and so differentiate between any contaminating genomic DNA. The RNA from the P30 gene, which codes for the P30 surface protein only found in the tachyzoite, would be a suitable target but it does not contain any introns (Burg *et al.* 1988). The genes which do contain introns and which could be considered include the α and ß tubulin, B1 and P28 genes.

In-situ PCR may provide information on the location of the parasite in different cell type, for example in tissue sections, or cytospins of blood cells. Currently *in-situ* hybridisation has been used to examine single cells for a range of organisms in wide variety of samples (Foley *et al.* 1993). This would distinguish between the presence of DNA in tissue cysts and tachyzoites and therefore indicate whether the parasite was active or latent. *In-situ* hybridisation can also be combined with RT qPCR (Foley *et al.* 1993) which may provide further information on the location and activity of the parasite.

7.6 CONCLUDING REMARKS

The PCR has been demonstrated to be a sensitive and specific method of detecting *T. gondii* DNA. It is potentially a valuable tool in the diagnosis of infection in patients where conventional testing is of limited value. This study shows the B1 PCR to be the system of choice due to its superior sensitivity. However there were several problems associated with the system such as the risks of sampling error, and the detection of parasite in a small number of patients who were clinically well. Therefore the B1 PCR

should be used in combination with other systems such as MRI and CAT scans, and the presence of other pathogens should be negated. There was no evidence of false positive results in either the patient or animal studies using this system, so any future positive results in a clinical setting must be taken seriously and patients should subsequently be closely monitored. Quantitative PCR may also reveal whether the DNA detected is at a level likely to result in illness, and would be an ideal way to monitor the progress of infection. It may also indicate whether therapy would successfully prevent recrudescence in patients who are at particular risk.

The animal and patient studies revealed that parasitaemia was present in both primary infection and in recrudescence of a chronic infection. The duration of parasitaemia was considerably shorter in cases of recrudescence than in primary infection, but as it occurred well before clinical signs of illness, therapy may be considered prior to any clinical signs of encephalitis. Until more appropriate drugs are available which would specifically treat the tissue cyst, the presence of parasite at any level should warrant the initiation of conventional therapy alongside careful management of the patient.

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APPENDIX I

Suppliers

Amersham International Plc., Amersham Place, Little Chancelot, Buckinghamshire, UK. HP7 9NA.

Bio 101 Inc., PO Box 2284, LaJolla, CA., 92038 USA.

Bio-Rad Laboratories Ltd., Bio-Rad House, Maylands Avenue, Hemel Hempstead, Hertfordshire, UK. HP2 7TD.

Boehringer Mannheim, Bell Lane, Lewes, East Sussex UK. BN7 1LG.

Cambio, 34 Millington Road, Cambridge, UK. CB3 9HP.

Chemicon International Plc., (agents for Quadratech) 27515 Enterprise Circle West, Temecula, CA., 92590 USA.

Gibco Life Technologies Ltd., PO Box 35, Trident House, Renfrew Road, Paisley, Strathclyde, UK. PA3 4EF.

Invitrogen, R and D Systems Europe Ltd., 4-10 The Quadrant, Barton Lane, Abingdon, UK. OX14 3YS.

Nycomed, (Division Diagnostica) PO Box 4284, Torshov N-0401, Oslo 4, Norway.

Oswell DNA Services, Department of Chemistry, University of Edinburgh, Kings Buildings, West Mains Road, Edinburgh, UK. EH9 3JJ.

Pharmacia Biosystems Ltd., Davy Avenue, Knowlhill, Milton Keynes, UK. MK5 8PH

Promega, Delta House, Enterprise Road, Chulworth Research Centre, Southampton, UK. SO1 7NS.

Quadratech, see Chemicon International Plc.

Scotlab Ltd., Kirkshaws Road, Coatbridge, Strathclyde, UK. ML5 8AD.

Sigma, Fancy Road, Poole, Dorset, UK. BH17 7BR.

Stratagene Ltd., Cambridge Innovation Centre, Cambridge Science Park, Milton Road, Cambridge, UK. CB4 4GF.

APPENDIX II

Published Results

PUBLISHED PAPER NOT INCLUDED