Molecular diagnosis of infection with *Toxoplasma gondii* in immunocompromised patients

Benjamin Edvinsson

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ABSTRACT

The protozoan parasite *Toxoplasma gondii* infects more than a billion people worldwide. The parasite is generally divided into three clonal lineages, denoted type I, II and III. Primary infections in humans are usually asymptomatic or characterized by non-specific symptoms, and are followed by latent chronic infection, which is normally harmless to the host. However, if the immune system is suppressed, as in bone marrow transplantation (BMT) or human immunodeficiency virus (HIV) infection, reactivated toxoplasmosis or primary infection may cause severe disease. Molecular methods are the primary tools to diagnose toxoplasmosis in immunocompromised patients. The molecular diagnosis would benefit from standardization and a risk profile to identify patients who should be tested for toxoplasmosis by the polymerase chain reaction (PCR).

The present thesis shows that real-time PCR preceded by manual or automated DNA extraction of pure parasites could be used to detect *T. gondii* DNA corresponding to one parasite genome in a reaction volume. Different detection limits were however obtained when whole blood samples were spiked with parasites, and analyzed with conventional PCR, nested PCR oligochromatography, real-time PCR SYBR green, or real-time PCR TaqMan. The results show that DNA extraction methods and PCR assays may perform differently depending on the type of sample.

A comparative study of two real-time PCR targets, a 200-300-fold repeated 529 bp element, and the 35-fold repeated *B1* gene, showed that *T. gondii* DNA was detected more sensitively and more accurately when a more repeated PCR target was used. Furthermore, the performance of real-time PCR targeting the 529 bp element was not affected when an internal amplification control was included.

A rapid and highly reproducible Pyrosequencing assay was developed, which is a promising method for routine use to discriminate between type I, II and III *T. gondii* strains in clinical samples. Identification of *T. gondii* strains may help to increase our knowledge about the significance of the genotypes in human toxoplasmosis.

A prospective study of the magnitude of toxoplasmosis in BMT recipients, performed by PCR and real-time PCR analysis of peripheral blood samples, showed that it was not possible to identify a specific risk profile associated with an increased risk for toxoplasmosis. A retrospective study of pulmonary toxoplasmosis in immunocompromised HIV patients, performed by real-time PCR analysis of bronchoalveolar lavage samples, confirmed that a particular risk profile could not be identified. However, it seems important that surveillance by PCR is initiated immediately after allogeneic BMT in seropositive patients, and the conclusion was made that a PCR test for *T. gondii* should be performed if pulmonary symptoms of unknown aetiology occur in immunocompromised HIV positive patients. After review of medical records we suggest that toxoplasmosis may be suspected if ocular infections of unknown aetiology occur in seropositive allogeneic BMT patients and HIV patients, and that asymptomatic and symptomatic toxoplasmosis may occur despite trimethoprim/sulfamethoxazole prophylaxis. We suggest that monitoring of seropositive immunocompromised patients using molecular methods may serve multiple purposes; i) to diagnose toxoplasmosis at an early stage to facilitate effective treatment, ii) to correlate the parasitic burden to clinical symptoms, iii) to further investigate the significance of the genotype of the parasite, and iv) to evaluate the ability of prophylactic regimens to prevent reactivations and full-blown toxoplasmosis.
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LIST OF ABBREVIATIONS

AIDS  Acquired immunodeficiency syndrome
APC   Antigen presenting cell
BMT   Bone marrow transplantation
cIAC  Competitive internal amplification control
CMI   Cell mediated immunity
CNS   Central nervous system
CP    Crossing point
C_t   Cycle threshold
ds-DNA Double stranded DNA
HIV   Human immunodeficiency virus
IAC   Internal amplification control
IFN-γ Interferon gamma
IL    Interleukine
nIAC  Non-competitive internal amplification control
NK cell Natural killer cell
PCR   Polymerase chain reaction
SNP   Single nucleotide polymorphism
T_H1 T helper type 1 cell
T_H2 T helper type 2 cell
TMP/SMX Trimethoprim/sulfamethoxazole
1 INTRODUCTION TO TOXOPLASMA GONDII

The first known observation of an organism with an appearance like *Toxoplasma gondii* was made in 1900 by Laveran, who observed the organism in spleen and bone marrow of Java sparrows (Laveran 1900; Ho-Yen and Joss 1992; Tenter, Heckeroth *et al.* 2000). A more definite description was made in 1908, by Nicolle and Manceaux, who found the parasite in blood, spleen, and liver of the North African rodent *Ctenodactylus gondii* (Nicolle and Manceaux 1908). A year later they proposed a new species name, *Toxoplasma gondii*, based on the appearance of the parasite; *Toxoplasma*, from the Greek terms toxon (= bow), and plasma (= form).

The first case of congenital toxoplasmosis, including ocular disease, was probably observed in 1923 (Ho-Yen and Joss 1992; Tenter, Heckeroth *et al.* 2000). Early descriptions of “toxoplasma-like” organisms do however remain uncertain due to the similarities between *T. gondii*, Sarcosporidia and Encephalitozoon when microscopically examined. More than a decade elapsed before *T. gondii* was recognized as the causative agent for neurological disorders in newborns, nowadays referred to as “congenital toxoplasmosis”. Toxoplasmosis in immunocompromised patients was described in 1953, in a patient with Hodgkin’s disease, and in the 1960s, in patients with cancer (Ho-Yen and Joss 1992; Tenter, Heckeroth *et al.* 2000). The life-cycle of *T. gondii* was described in 1969 (Dubey, Miller *et al.* 1970).

Today we know that *T. gondii* is an obligate intracellular parasite that penetrates, multiplies, and survives within host cells. We know that the parasite has low host specificity, that it infects warm-blooded animals all over the world, and also that most human infections are asymptomatic and harmless (Hill, Chirukandoth *et al.* 2005). Still, there are patient groups in which toxoplasmosis may cause severe disease (Hill and Dubey 2002; Montoya and Liesenfeld 2004). The outcome of the disease is however constantly improved by the development and refinement of the diagnostic procedures and the treatment protocols.

1.1 Life cycles

The life cycle of *T. gondii* includes two phases; the enteroepithelial and the extraintestinal cycles (Dubey, Lindsay *et al.* 1998). During these cycles, the parasite exists in three infectious life forms; sporozoites contained in oocysts, bradyzoites contained in tissue cysts, and tachyzoites (Dubey, Lindsay *et al.* 1998; Dubey 2004; Montoya and Liesenfeld 2004). Transmission to susceptible
hosts usually occurs through the oral route, e.g. by accidental ingestion of oocysts from contaminated soil, vegetables, or fruit. The infection is also acquired by ingestion of tissue cysts contained in raw or undercooked meat (Cook, Gilbert et al. 2000; Tenter, Hecker Roth et al. 2000). Pork and lamb are commonly infected and carriers of T. gondii. In Sweden, around 17% of the adult swine population, and 10-45% of sheep were found to be carriers of the parasite (Lunden, Nasholm et al. 1994; Lunden, Lind et al. 2002). Tissue cysts are destroyed by heating to >65°C, when meat turns from pink to grey, or by freezing to.

1.2 Enteroepithelial cycle

Cats (wild or domesticated) are the definite hosts for T. gondii (Klaren and Kijlstra 2002; Hill, Chirukandoth et al. 2005). The enteroepithelial cycle (also known as the sexual or intestinal cycle) occurs exclusively in these animals. Cats are usually infected by ingestion of parasites contained in tissue cysts of infected intermediate hosts, such as birds and rodents (Tenter, Hecker Roth et al. 2000). Upon digestion, the proteolytic enzymes in the cat’s stomach and small intestine degrade the tissue cysts. Bradyzoites, which are a slow multiplying form of the parasite, are released, and penetrate the intestinal epithelial cells of the cat where they begin to multiply. A sexual reproduction occurs. Millions of oocysts are produced and shed in the cat’s feaces during a limited period of time, about 2-3 weeks (Montoya and Liesenfeld 2004). Oocysts are very resistant to degradation by environmental factors, and may remain infectious for a year or more (Dumetre and Darde 2003).

1.3 Extraintestinal cycle

The extraintestinal cycle (also known as the asexual cycle) takes place in intermediate hosts, and also in cats, following a primary infection (Dubey, Lindsay et al. 1998; Black and Boothroyd 2000). Depending on the source of infection, sporozoites released from oocysts, or bradyzoites released from tissue cysts, penetrate the epithelial cells of the intestine. Transformation into the tachyzoite form occurs, and the parasite multiplies and disseminates through the blood stream or via the lymphatic system. Tachyzoites may enter any nucleated cell, where they multiply until the cell is disrupted (Black and Boothroyd 2000). Released tachyzoites enter new host cells, and the process is repeated.

Toxoplasmosis may involve the central nervous system (CNS), the eye, muscle tissue, and placenta (Singh and Husain 2000; Jones, Lopez et al. 2001; Filisetti and Candolfi 2004; Bonfioli and Orefice 2005). As the host develops an immune
response, tachyzoites transform into bradyzoites contained in tissue cysts, which may have a life long persistence in the host (Montoya and Liesenfeld 2004). The parasite is transferred to new hosts upon ingestion of tissue cysts, for example by a predator.

Fig 1: An overview of the life cycle of *T. gondii*. Cats are the definitive hosts of *T. gondii* and are infected by consumption of infected prey, or by oocysts. Oocysts are shed by infected cats, and can infect intermediate hosts such as mammals, birds, and humans. After primary infection, tachyzoites transform into bradyzoites, which are contained in tissue cysts. Intermediate hosts may also be infected by ingestion of infected meat.

1.4 Epidemiology

It has been estimated that a third of the world population is infected with *T. gondii* (Hill, Chirukandoth et al. 2005). Environmental conditions, differences in the type of food consumed, animal species used in the food industry, and the number of cats are examples of factors that may influence the spread of the parasite. Water-borne transmission of *T. gondii* has earlier been considered uncommon, but recently human outbreaks connected to water reservoirs have
been reported (Aramini, Stephen et al. 1999; Palanisamy, Madhavan et al. 2006).

The prevalence of human infection varies between different countries, and is higher in temperate and tropical areas and at lower altitudes than in areas with a colder climate and at higher altitudes (Bonfioli and Orefice 2005; Sukthana 2006). In the United States and the United Kingdom, the overall prevalence is estimated to 16-40%, and in continental Europe and South America the corresponding figures are 50-80% (Joynson 1992; Jones, Kruszon-Moran et al. 2001; Hill and Dubey 2002). The prevalence in pregnant women in Denmark was found to be 27%, and in Sweden the corresponding figures were 26% and 14% in two distinct geographic areas (Lebech, Larsen et al. 1993; Evengard, Petersson et al. 2001). The birth prevalence of congenital toxoplasmosis ranges from 0.17 to 0.73% in Scandinavia (Lappalainen, Koskela et al. 1992; Lebech, Larsen et al. 1993; Jenum, Stray-Pedersen et al. 1998; Evengard, Petersson et al. 2001). Toxoplasmosis often remains undiagnosed, and the exact source of the infection is usually not determined (Montoya and Liesenfeld 2004). At present it is not possible to tell from which form of the parasite (tissue cysts or oocysts) an infection was acquired (Hill and Dubey 2002).

1.5 Strains

Isoenzyme profiles of T. gondii isolates have suggested that the T. gondii population is divided into different strains (Darde, Bouteille et al. 1988). These results were later confirmed by analysis of multiple genetic markers, and it seems as if the majority of the T. gondii strains isolated in Europe and North America fall into three distinct clonal lines, denoted type I, II and III (Sibley and Boothroyd 1992; Howe and Sibley 1995; Darde 1996; Grigg, Bonnefoy et al. 2001; Ajzenberg, Banuls et al. 2004). Variations within the strain types are very rare, which suggests that type I, II, and III have undergone clonal expansion (Su, Evans et al. 2003). Less than 1% of the isolates contain highly polymorphic DNA sequences, and are defined as “exotic” or “atypical” (Ajzenberg, Banuls et al. 2004). Interestingly, atypical and exotic strains have alleles that are found in type I and III strains, but rarely from type II (Boothroyd and Grigg 2002).

Meiosis, which occurs during the enteric epithelial cycle, may potentially give rise to different genotypes. Meiotic recombination has, however, not been considered as a major source of genetic variation and diversity. A possible explanation to the low diversity is that definite and intermediate hosts, which are subjected to primary infections, continuously select for the persistence of the most successful genotypes. In addition, the parasite is frequently transmitted by ingestion of tissue cysts, and sexual recombination does not occur (Su, Evans et
There are few reports that describe infections in definite hosts with different strains involved (Ajzenberg, Banuls et al. 2004).

Generally, strains that are highly virulent in mice are also highly virulent in another animals, but there are differences in virulence depending on host species (Sibley and Boothroyd 1992; Howe and Sibley 1995). For example, laboratory tests have shown that infection with the RH strain in mice is lethal, while rats showed a peak in the parasite load in different organs followed by a complete disappearance of parasites (Dubey, Shen et al. 1999; Zenner, Foulet et al. 1999). The virulence attributed to a certain strain of *T. gondii* seems to depend on host genetics in association with the strain phenotype. Virulence is also closely related to the status of the immune system of the host. A previously latent and asymptomatic infection may reactivate with fatal consequences if immunosuppression is introduced.

Type II *T. gondii* is frequently associated with human toxoplasmosis, although type I and atypical strains have been isolated in more severe cases of toxoplasmosis (Howe and Sibley 1995; Howe, Honore et al. 1997; Darde 2004). Surprisingly, a Spanish study showed that type II strains dominated in Acquired Immunodeficiency Syndrome (AIDS) patients, but type I strains were most prevalent in congenital infections (Fuentes, Rubio et al. 2001). Furthermore, cases of ocular infections in Brazil have been associated with type I and atypical strains (Vallochi, Nakamura et al. 2002; Jones 2006; Khan 2006). In the same country, only type I and III have been isolated from chicken, which is a main food source (Dubey, Graham et al. 2003; Dubey, Gennari et al. 2006). Strain types involved in subclinical infections are however rarely identified due to the lack of appropriate techniques.

### 1.6 Immune response to *T. gondii*

*T. gondii* tachyzoites are highly virulent. For the survival of the host it is important that their proliferation and spread is controlled (Frenkel 1988). Host defense mechanisms against infection with *T. gondii* consist of innate immunity, which mediates the initial protection, and adaptive immunity, which is divided into humoral and cell-mediated immunity (Bhopale 2003; Filisetti and Candolfi 2004).

#### 1.6.1 Innate immune response

Macrophages, natural killer (NK) cells, and cytokines are the main components involved in the innate immune response against *T. gondii* (Bhopale 2003;
Filisetti and Candolfi 2004). In the acute phase of the infection, tachyzoites stimulate macrophages to produce the cytokine interleukine (IL) 12. IL-12 acts on NK cells, which start to produce interferon gamma (IFN-γ) (Sher, Oswald et al. 1993; Gazzinelli, Wysocka et al. 1994). IFN-γ stimulates macrophage killing of phagocytosed tachyzoites, and is a major mediator of host resistance during the acute and chronic phases of *T. gondii* infection (Suzuki, Orellana et al. 1988; Sibley, Adams et al. 1991; Johnson 1992; Yap and Sher 1999). Thus macrophages and NK cells function cooperatively to destroy tachyzoites, and to minimize the spread of the parasite. IFN-γ also induces expression of co-stimulatory molecules on antigen presenting cells (APCs), such as macrophages.

**1.6.2 Adaptive immune response**

The adaptive immune response to *T. gondii* can be divided in cell-mediated immunity and humoral immunity. The cell-mediated immunity is an essential component of the host’s immune response to *T. gondii* infection (Lindberg and Frenkel 1977; Denkers and Gazzinelli 1998). Acquired immunity to *T. gondii* infection is characterized by a strong CD4+ and CD8+ T-lymphocyte activity (Gazzinelli, Hakim et al. 1991; Parker, Roberts et al. 1991; Denkers and Gazzinelli 1998).

As mentioned above, IFN-γ stimulated APCs express co-stimulatory molecules, and also display *T. gondii* peptide antigens via their major histocompability complex (MHC) class II molecules (Denkers and Gazzinelli 1998). Naïve CD4+ T lymphocytes recognize *T. gondii* peptide antigens, begin to differentiate, and undergo clonal expansion. Macrophages, and other APCs, secrete IL-12 which acts on the naïve CD4+ cells and drives their differentiation into a TH1 effector cell subset (Filisetti and Candolfi 2004). The derived TH1 cell population secretes a cytokine profile which induces an inflammatory response, and stimulates anti-toxoplasma antibody production. The TH1 cell population is also an important source of further IFN-γ release. Naïve CD8+ T cells, on the other hand, recognize MCH class I bound *T. gondii* antigens, and in combination with IL-12 induced co-stimulatory signals they are activated and differentiate into CD8+ T lymphocytes with cytolytic activity (Denkers and Gazzinelli 1998). Cytolytic CD8+ lymphocytes recognize and destroy tachyzoite-containing cells, and the reservoirs of infection during the acute phase are thereby destroyed (Hakim, Gazzinelli et al. 1991; Subauste, Koniaris et al. 1991).

Although the cell-mediated immunity limits the spread of the infection, the immune responses need to be regulated in order to avoid host damage caused by an exaggerated inflammatory response. IL-10, transforming growth factor β, IL-
5, and IL-4 are factors that balance T_{H1} cytokine release (Denkers and Gazzinelli 1998; Bhopale 2003).

The humoral immune response involves B-lymphocytes and the production of anti-toxoplasma antibodies. Naïve B-cells undergo clonal expansion and differentiate into antibody secreting effector cells as they recognize antigens. T helper cells may stimulate this process. The humoral immune response appears to play a minor role for the host’s ability to control \textit{T. gondii} infection, but is essential for the serological diagnosis of toxoplasmosis in humans (Reyes and Frenkel 1987; Filisetti and Candolfi 2004; Lappalainen and Hedman 2004).

### 1.6.3 Immune response to chronic infection

When host immunity to \textit{T. gondii} infection has been acquired, the parasite transforms from the tachyzoite form into bradyzoites. Bradyzoites are contained in intracellular cysts, and are thereby protected from the host’s immune system. Different hypothesis have been postulated about how the parasite replication is controlled by the immune system during the chronic phase (Denkers and Gazzinelli 1998). One theory suggests that the immune system actively induce conversion of tachyzoites into bradyzoites, by nitric oxide produced by IFN-\gamma activated macrophages (Bohne, Heesemann \textit{et al.} 1994; Denkers and Gazzinelli 1998). Furthermore, it has been proposed that secretion of IFN-\gamma affects the transformation of the rapidly proliferating tachyzoites into bradyzoites, and that IFN-\gamma suppresses cyst rupture and release of parasites (Suzuki, Orellana \textit{et al.} 1988; Suzuki, Conley \textit{et al.} 1989; Bohne, Heesemann \textit{et al.} 1993). A second theory suggests that the immune system is active only against parasites that are released during cyst rupture, but does not have an effect on encysted bradyzoites.
2 CLINICAL MANIFESTATIONS

The outcomes of toxoplasmosis depend on many factors, e.g. host characteristics, function of the immune system, type of strain, diagnosis, and treatment. As mentioned previously, most primary infections that occur in immunocompetent individuals are asymptomatic, or may cause a mononucleosis-like illness with headache, fever, malaise, and lymphadenopathy. From an evolutionary point of view, the parasite seems to be well adapted to cope with the human immune system, and to cause minimal damage to its host. There are, however, clinical settings in which toxoplasmosis can cause serious pathology: congenital toxoplasmosis following acquired infection in pregnant women, ocular toxoplasmosis in immunocompetent patients, and toxoplasmosis in immunocompromised patients.

2.1 Congenital toxoplasmosis

A developing fetus may be congenitally infected with *T. gondii* if the mother acquires the infection during gestation. Different clinical pictures are known, involving retinochoroiditis, miscarriage, mental retardation, microcephaly, hydrocephalus, or seizures (Hill and Dubey 2002; Kravetz and Federman 2005). The severity of congenitally acquired toxoplasmosis is related to the time of the primary infection; more severe symptoms are observed if the infection is acquired during the first trimester of gestation than during the second or third (Bonfioli and Orefice 2005; Kravetz and Federman 2005). It also seems as if there is a connection between the quantity of parasites found in amniotic fluid and the severity of the disease (Costa, Ernault et al. 2001; Romand, Chosson et al. 2004). The social and economic impacts of congenital toxoplasmosis are enormous (Roberts, Murrell et al. 1994).

Diagnosis of congenital toxoplasmosis is sometimes challenging because of the asymptomatic course of infection in immunocompetent individuals. In some cases, it is hard to discriminate a recent acute infection from an infection acquired in the past, which has little or no significance for the unborn child. With a timely diagnosis, and antibiotic therapy started early, the rate of sequel is reduced. Information about congenital toxoplasmosis in Swedish can be found on www.infpreg.com.
2.2 Ocular toxoplasmosis

Ocular toxoplasmosis, and development of retinochoroiditis, usually originates from a congenital infection and may cause ocular disease in immunocompromised and immunocompetent individuals (Rodgers and Harris 1996; Sasaki, Arana et al. 2000). It is the most common cause of human retinochoroiditis (Gilbert, Dunn et al. 1999). Ocular toxoplasmosis may also occur following acquired infection later in life (Jones, Muccioli et al. 2006; Palanisamy, Madhavan et al. 2006). As mentioned previously, an unusually high prevalence of severe cases of acquired ocular toxoplasmosis has been reported from Brazil. Interestingly, atypical strains have been associated with such severe ocular disease (Glasner, Silveira et al. 1992; Grigg, Ganatra et al. 2001; Vallochi, Nakamura et al. 2002; Vallochi, Muccioli et al. 2005).

2.3 Toxoplasmosis in HIV infected patients

If immunosuppression is introduced, as in transplant recipients or in patients with AIDS, an opportunistic reactivation of a latent T. gondii infection might occur, often with a fatal outcome. The connection between toxoplasmosis affecting the CNS and HIV infection is today well established (Luft and Remington 1992; Hill and Dubey 2002). Encysted bradyzoites are often found in large numbers in the CNS of patients with AIDS, and it is therefore not surprising that the incidence of toxoplasmic encephalitis is high in this patient group (Denkers and Gazzinelli 1998). About 10-50% of HIV patients chronically infected with T. gondii develop toxoplasmic encephalitis (McCabe and Remington 1988; Richards, Kovacs et al. 1995).

In advanced HIV, when CD4+ T lymphocyte count is below 200 cells/mm³, the incidence of toxoplasmosis increase (Jung and Paauw 1998). A decreased IL-12 secretion may also be a contributing factor to the increased incidence. A synergetic relationship between the presence of tachyzoite products and the replication of HIV-1 virus has been suggested. Toxoplasmosis enhances viral replication, and the viral replication itself decreases host immunity against T. gondii infection (Bala, Englund et al. 1994). CNS lesions are commonly seen in HIV patients, which is the result of tissue destruction caused by an uncontrolled proliferation of tachyzoites rather than immunopathologic changes due to an inflammatory response (Denkers and Gazzinelli 1998). Pulmonary infections and ocular toxoplasmosis may also be expected (Rodgers and Harris 1996; Campagna 1997; Petersen, Edvinsson et al. 2006).
2.4 Toxoplasmosis in transplant recipients

Toxoplasmosis is a rare but severe complication in transplant recipients. The disease has been diagnosed after e.g. heart, liver, kidney, bone marrow (BMT), and stem cell transplantations (Da Cunha, Ferreira et al. 1994; Gallino, Maggiorini et al. 1996; Renoult, Georges et al. 1997; Lappalainen, Jokiranta et al. 1998; Mele, Paterson et al. 2002). In most cases of toxoplasmosis after BMT, the disease is the result of an opportunistic reactivation in the context of impaired cell mediated immunity (Derouin, Gluckman et al. 1986; Derouin, Devergie et al. 1992). The disease may also be the result of a primary infection transferred by a transplanted solid organ harboring the parasite, or from contaminated blood transfusions (Lappalainen and Hedman 2004). The incidence of toxoplasmosis in BMT recipients is connected to the prevalence of T. gondii seropositivity in different countries, and ranges from 0.3-5% (Slavin, Meyers et al. 1994; Mele, Paterson et al. 2002).

A review of 110 cases of toxoplasmosis after BMT showed that the prognosis was poor; 66% of the patients died (Mele, Paterson et al. 2002). Unfortunately, post mortem diagnosis is common due to the often non-specific clinical signs of toxoplasmosis (de Medeiros, de Medeiros et al. 2001). The infection is often disseminated at the time of diagnosis, and may involve CNS, lungs, and heart (Derouin, Devergie et al. 1992; Mele, Paterson et al. 2002). Ocular infections may also occur as a complication in association with BMT (Peacock, Greven et al. 1995).

Toxoplasmosis is more prevalent among allogeneic transplant recipients (transfer of material from one person, the donor, to another person, the recipient) than in those receiving autologous transplants (transfer of material within the donor), certainly due to differences in immunosuppressive regimens in these two procedures (de Medeiros, de Medeiros et al. 2001). Prospective studies of the magnitude of toxoplasmosis in transplant recipients, by PCR analysis of peripheral blood samples, have suggested that reactivated T. gondii infections occur more frequently after transplantation than what was previously thought (de Medeiros, de Medeiros et al. 2001; Martino, Bretagne et al. 2005). No specific risk profile has been identified that would help to predict and aid timely diagnosis of the disease in transplant recipients. Toxoplasmosis should however be suspected in seropositive patients with headache, fever, or cerebral symptoms of unexplained cause (Bhopale 2003).
2.5 Treatment

Tachyzoites, which are highly proliferative and metabolic active, can be effectively reduced in number by proper treatment. There is, however, little evidence that encysted bradyzoites can be eradicated with anti-parasite drugs, although animal studies suggest that certain drugs may have an effect (Huskinson-Mark, Araujo et al. 1991; Petersen and Schmidt 2003). The rationale for treatment of acute toxoplasmosis is therefore to inhibit the proliferation of tachyzoites to reduce the pathology, and sometimes to administer corticosteroids to reduce the inflammatory response. In congenital infections, treatment may reduce the number of tissue cysts, which in turn may reduce the risk for reactivation later in life based on the theory that tissue cysts ruptures spontaneously over time (Petersen and Schmidt 2003).

The effectiveness of sulfonamides against *T. gondii* was described in 1942, and in 1953 the synergy of combining sulphadiazine with pyrimethamine was detected (Petersen and Schmidt 2003). A combination of pyrimethamine and sulfadiazine is still used to treat toxoplasmosis, although alternative treatment protocols have been developed.

For example, ocular toxoplasmosis can be treated with combinations of pyrimethamine, sulfadiazine, corticosteroids, and folinic acid (Bonfioli and Orefice 2005). Clindamycin, spiramycin, azithromycin, or atovaquone can be used in cases where the patient is intolerant to sulfadiazine (Bonfioli and Orefice 2005). Therapy regimens during pregnancy may consist of spiramycin, sulfadiazine, pyrimethamine, and folinic acid. Newborns with toxoplasmosis are usually treated with pyrimethamine, sulfadiazine and folinic acid to reduce the risk for ocular toxoplasmosis (Bonfioli and Orefice 2005). Toxoplasmosis in the immunocompromised patients can also be treated with these combinations. Prophylactic regimens consist of trimethoprim/sulfamethoxazole (TMP/SMX), sulfadiazine, clindamycin, pyrimethamine, and folinic acid (Bonfioli and Orefice 2005).
3 DIAGNOSIS

Diagnosis of toxoplasmosis in humans is performed using different techniques. A few examples of these techniques are mouse inoculation, detection of anti-toxoplasma antibodies, histological demonstration of tachyzoites in tissue sections or smears of body fluid, and detection of *T. gondii* DNA by molecular methods (Montoya 2002; Lappalainen and Hedman 2004; Remington, Thulliez et al. 2004).

3.1 Serological techniques

Detection of anti-toxoplasma antibodies indicates that a person has been infected with *T. gondii* some time in the past. There are many different serological techniques available, e.g. the Sabin-Feldman dye test, the indirect hemagglutination assay, the indirect fluorescence antibody assay, the latex agglutination test, the enzyme-linked immunosorbent assay, and the immunosorbent agglutination assay test (Hill and Dubey 2002; Montoya 2002).

In asymptomatic infections, the only indication of a primary infection is seroconversion indicated by anti-toxoplasma IgM or IgG antibodies. IgM antibodies usually become detectable within days after infection, while IgG antibodies become detectable after 1-2 weeks and may have lifelong persistence. The presence of IgM antibodies indicates a recent infection, but the tendency of IgM to remain detectable for a long time has been demonstrated (Lappalainen and Hedman 2004; Petersen, Borobio et al. 2005). Affinity is a measure of the attraction between an antigen and an antibody. Avidity, defined by Lappalainen et al as functional affinity, or the net antigen binding force of populations of antibodies, is usually low for IgG shortly after a primary infection, and increases over time (Jenum, Stray-Pedersen et al. 1997; Lappalainen and Hedman 2004). Avidity is used to discriminate a recently acquired infection from an infection acquired some time in the past.

3.2 Molecular methods

A milestone in the field of molecular biology was set in the 1980s with the development of the polymerase chain reaction (PCR) (Saiki, Scharf et al. 1985; Saiki, Gelfand et al. 1988). The PCR is today frequently used to detect *T. gondii* DNA in clinical samples (Bretagne, Costa et al. 2000; Reischl, Bretagne et al. 2003; Jalal, Nord et al. 2004; Martino, Bretagne et al. 2005; Edvinsson, Lappalainen et al. 2006; Petersen, Edvinsson et al. 2006). PCR is performed by
direct detection of parasite DNA, and the results do not depend on the immunological status of the patient.

Since its introduction PCR has been very much refined, and at present low amounts *T. gondii* DNA can be detected (Reischl, Bretagne *et al.* 2003; Edvinsson, Jalal *et al.* 2004; Jalal, Nord *et al.* 2004; Edvinsson, Lappalainen *et al.* 2006). The sensitivity and specificity of the PCR depend on multiple factors, such as the DNA extraction protocol, the characteristics of the DNA sequence that is amplified, and the optimization of the reaction conditions. The main problem with PCR used to detect *T. gondii* is the lack of a standardized protocol (Bastien 2002; Martino, Bretagne *et al.* 2005). Three different PCR principles for detection of *T. gondii* DNA are described below: conventional PCR, PCR oligochromatography, and real-time PCR.

### 3.2.1 Conventional PCR

The PCR is based on the same principles for copying DNA as those found in nature. The building blocks of DNA are four nucleotides, represented by the letters A (adenine), C (cytosine), G (guanine), and T (thymine). DNA is double stranded, and an A on one strand always pairs with a T on the other, whereas C always pairs with G. In brief, PCR is performed by denaturation of double stranded DNA to generate single stranded DNA molecules. Primers, which are short single stranded oligonucleotides, anneal to the single stranded DNA molecules at specific locations. The design of the primers determines which DNA sequence that will be amplified. Next, a polymerase enzyme recognizes annealed primers and initiates elongation. Double stranded (ds) DNA molecules are synthesized. The process is repeated in cycles and the amount of double stranded DNA copies increases exponentially for each cycle. The large amount of copies of a specific DNA fragment are usually analyzed using an agarose gel, and can be visualized by staining with ethidium bromide and illumination with ultra violet light.

### 3.2.2 PCR oligochromatography

A commercially available kit for detection of *T. gondii* is based on PCR followed by oligochromatography (Edvinsson, Jalal *et al.* 2004). The principle of oligochromatography of PCR products is that a specific probe recognizes the PCR product, which gives rise to a red band on an oligochromatography strip (Fig 2). Oligochromatography reduces the time needed for post PCR analysis,
and increases the specificity of the PCR by utilizing specific probe hybridization.

![Diagram of the chromatography stick](image)

**Fig 2:** Oligochromatography of PCR products. A conjugate pad contains dried colloidal gold-conjugated probes that recognize the PCR product. The chromatographic stick also contains a line of coated anti-hapten antibodies. The PCR products are detected by dipping the oligochromatography sticks into the PCR reaction mixture. The reaction mixture migrates towards the upper absorbant of the stick by capillary force. The PCR products react with the colloidal gold-conjugated probe, and accumulate on the line where the anti-hapten antibody is coated. The reaction gives rise to a red band for positive samples in less than 5 min.

### 3.2.3 Real-time PCR

Conventional PCR is based on end-point detection of amplified products, and it is not possible to monitor the reaction from start to end in order to quantify the amount of starting material, or to calculate the efficiency with which amplification occurs. The first attempts to monitor amplification of PCR products in real-time were performed using a video camera and inclusion of ethidiumbromide in the PCR mixture (Higuchi, Fockler *et al.* 1993). The emitted fluorescence was found to be proportional to the amount of double stranded products formed during the PCR. A cycle number at which the fluorescence signal significantly exceeded the background signal could be identified and used to calculate the amount of starting material in sample.
The specific cycle number at which the fluorescence signal first exceeds the level of background fluorescence (calculated as ten-times the standard deviation of the average signal of the baseline) is today defined as “crossing point” (CP), or "cycle threshold” (Ct) (Arya, Shergill et al. 2005). CP of dilution series of a known amount of target DNA copies can be plotted in a standard curve from which the amount of starting material in unknown samples, amplification efficiency, and error can be calculated.

Fig 3: The nomenclature commonly used in quantitative real-time PCR includes A) baseline, the fluorescence signal of the reporter molecules in their quenched/un-excited state B) cycle threshold (Ct) or crossing point (CP) C) Log/exponential phase D) the plateau phase.

The exponential rate with which PCR products are produced can be described by the following equation:

\[ N_n = N_0 \times (1 + E)^n \]

\(N_n\) is the number of DNA molecules after \(n\) cycles, \(N_0\) is the number of DNA molecules before PCR is initiated, and \(E\) is the amplification efficiency. The amplification efficiency ranges from 0 to 2 when a LightCycler instrument is used. Two is considered as a theoretical optimum (Reischl, Bretagne et al. 2003). The error is the mean squared error of a standard curve. It is calculated based on the number of data points used when creating a standard curve, and the
vertical distances between the data points and the regression line. The error value describes tube-to-tube variations, for example caused by pipetting errors (Edvinsson, Lappalainen et al. 2006).

Real-time PCR is currently performed using different platforms, e.g. LightCycler (Roche Diagnostics GmbH, Mannheim, Germany), ABI (Applied Biosystems, Foster City, CA, USA), iCycler iQ (Bio-Rad, Philadelphia, PA, USA), or the Smart Cycler Systems (Cepheid, Sunnyvale, CA, USA), each of which has its own characteristics. There are different chemistries available for detection of amplified products; fluorescent probes (dual hybridization probes, Scorpions, molecular beacons, hydrolysis probes), and double stranded (ds) DNA intercalating agents (SYBR green, ethidiumbromide) (Wilhelm and Pingoud 2003; Arya, Shergill et al. 2005; Espy, Uhl et al. 2006). The principles of two chemistries, SYBR green and TaqMan, are described in the following sections.

**Fig 4:** The principle of SYBR green. **A)** SYBR green molecules are present in the reaction mixture, but do not bind to single stranded DNA templates. The Taq polymerase (oval) is bound to an annealed primer and elongation is about to start. **B)** Elongation occurs and the SYBR green molecules bind to the double stranded DNA that is produced. **C)** An increase in fluorescence occurs.
3.2.4 SYBR green

SYBR green is one of the most frequently used real-time PCR chemistries. The dye binds to the minor groove of ds-DNA. Its affinity to ds DNA is more than 100-fold than that of ethidium bromide, and when bound, the fluorescence signal increases about 1000-fold (Fig 4) (Wilhelm and Pingoud 2003).

SYBR green is cheap to use compared to hybridization probes. This chemistry can be used together with any primer pair, which is not the case with hybridization probes where specific primers and probes work in synergy. SYBR green has no particular sequence specificity. Hence, the specificity of the fluorescence signal relies on the design of the primers. So called primer-dimers may give rise to ds-DNA fragments, which are detected by SYBR green. Primer dimers, or co-amplification of two different DNA targets, can be identified using melting curve analysis (Fig 5) (Ririe, Rasmussen et al. 1997).

It is sometimes believed that non-specific amplification revealed by melting curve analysis is a SYBR green related problem which hampers the performance of the PCR. Indeed, a systematic error of the quantification by altered amplification efficiency, due to non-specific amplification which affects the performance, may also occur when hybridization probes are used. The main difference is that such non-specific amplification does not give rise to a fluorescence signal with sequence specific probes.

Fig 5: Melting curve analysis used for quality control of real-time PCR SYBR green. Temperature (x-axis) is plotted against fluorescence values (y-axis), obtained as the temperature is gradually increased. The continuous line shows melting curve analysis of amplified T. gondii DNA, and peaks at 89.5°C. The dotted line shows a different product, in this case amplified internal amplification control in a sample negative for T. gondii, and peaks at 87.5°C. The dotted line also shows formation of primer dimers, which melt at around 79°C.
3.2.5 TaqMan

A TaqMan probe is dual-labeled. In its 5’-end, the probe is labeled with a reporter fluorophore which emits light upon excitation. The 3’-end of the probe has a quencher molecule attached to it. Quenching and emission of fluorescence is distant depending, and ceases if reporter and quencher are separated (Fig 6) (Wilhelm and Pingoud 2003; Arya, Shergill et al. 2005). The system depends on a bi-molecular binding of a primer and a probe to generate a fluorescence signal. TaqMan probes can be labeled with different reporter fluorophores, and used to monitor co-amplification of different targets in the same reaction mixture (Edvinsson, Lappalainen et al. 2006). Differently labeled probes can also be used to detect mutations if one probe is designed to anneal with wild-type sequences, and the other recognizes a mutated sequence.

![Fig 6: A) The TaqMan probe is designed to anneal a few bases downstream of one of the primers. In its intact and quenched state, the quencher is in close proximity to the reporter fluorophore, which significantly decreases the fluorescence of the reporter. B) The probe is degraded by the 5’ to 3’ exonuclease activity of the Taq polymerase during the elongation phase of the PCR. Reporter fluorophore and quencher are separated and quenching ceases.](image)

3.3 PCR targets for detection of *T. gondii*

The *B1* gene, also referred to as the *B1* repeat, is a 2214 base pair (bp) sequence with unknown function that is repeated 35 times in the genome of *T. gondii*
(Burg, Grover et al. 1989; Jalal, Nord et al. 2004). Only a few polymorphic sites have been described. Thus the \textit{B1} gene seems to be a suitable PCR target for detection of the \textit{T. gondii} by PCR (Grigg and Boothroyd 2001). PCR assays targeting the \textit{B1} gene have been used extensively (van de Ven E Melchers W 1991; P. A. Jenum 1998; J-M. Costa 2000; Mei-Hui Lin 2000; Buchbinder, Blatz et al. 2003; Jalal, Nord et al. 2004; Switaj, Master et al. 2005; Shah Jalal Submitted). Recently, a 200-300-fold repeated 529 bp element of unknown function has been described in the genome of \textit{T. gondii} (Homan, Vercammen et al. 2000). It has been postulated that an increased analytical sensitivity is achieved when a repeated DNA element is amplified, although some studies suggest that a difference in analytical performance depending on the number of repeats does not exist (Wastling, Nicoll et al. 1993; Jones, Okhravi et al. 2000; Buchbinder, Blatz et al. 2003; Filisetti, Gorcii et al. 2003; Reischl, Bretagne et al. 2003; Edvinsson, Lappalainen et al. 2006).

3.4 Amplification control

Generally, a negative PCR result indicates that there was no microbial DNA in the sample. It can also indicate that the PCR was inhibited and malfunctioned. PCR failure may have a variety of causes, e.g. instrument failure, errors during preparation of the reaction mixture, degraded or non-functional reagents, organic solvents or other PCR inhibitory substances still present in the extracted DNA. An amplification control is usually used to identify such false negative results.

3.4.1 External amplification control

An amplification control which is external consists of a PCR performed in a separate reaction mixture in order to detect PCR failure. External amplification control can be performed by amplifying a different PCR target, by a different set of primers than used to amplify microbial DNA (Edvinsson, Jalal et al. 2004; Jalal, Nord et al. 2004). An alternative way may be to analyze control samples spiked with a known amount of microbial DNA.

3.4.2 Internal amplification control

An internal amplification control (IAC) is a non-target DNA sequence which is present in the same reaction mixture as in which microbial DNA is amplified (Edvinsson, Lappalainen et al. 2006). Amplification of IAC in negative samples can be detected by gel analysis if the size of the product is different from that of amplified microbial DNA, by differently labeled probes (Fig 7), or by melting
curve analysis using SYBR green (Fig 5). In general, there are two different types of internal amplification controls; a competitive IAC (cIAC) and a non-competitive IAC (Hoorfar, Malorny et al. 2004; Edvinsson, Lappalainen et al. 2006).

A cIAC is a PCR target that contains primer-binding sites identical to those found in the DNA of the microbe, but has a substituted DNA sequence in between. Hence, it is amplified by the same set of primers as used for amplification of microbial DNA (Hoorfar, Malorny et al. 2004; Edvinsson, Lappalainen et al. 2006). The concentration of the cIAC is critical when this strategy is used; excess amounts of cIAC may cause competition between amplification of microbial DNA and amplification of the cIAC. This may lower the efficiency with which T. gondii DNA is amplified, with a decreased analytical sensitivity as a result. The amount of cIAC can be kept at a small amount to avoid such undesirable competition.

Fig 7: Three samples analyzed for T. gondii DNA by real-time PCR. + = T. gondii positive sample, * = T. gondii negative sample, and ● = PCR inhibition. Left: Amplification of T. gondii DNA. Right: amplification of internal amplification control. In the sample denoted ● neither T. gondii DNA nor internal amplification control was amplified, which is a false negative result caused by inhibition of the PCR.

Several strategies exist to produce cIACs, e.g. cloning, PCR mutagenesis, or the use of synthesized oligonucleotides (Hoorfar, Malorny et al. 2004). Once produced, the cIAC may be inserted in a plasmid to increase its stability (Hoorfar, Malorny et al. 2004). A recombinant cIAC plasmid can easily be produced in large quantities if contained in bacterial cells.
A nIAC is a PCR target which is completely different from microbial DNA, and is amplified by a different set of primers. In practice, a nIAC can be any DNA that is added to the sample. Except for the concentration of the nIAC itself, the concentration of the nIAC primers can be balanced to minimize production of nIAC products in order to avoid undesirable competition. The use of cIACs and nIACs are compared in Table 1.

Table 1: Characteristics of competitive internal amplification control (cIAC) versus non-competitive internal amplification control (nIAC).

<table>
<thead>
<tr>
<th>cIAC</th>
<th>nIAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>A cIAC with the same primer binding sites as in microbial DNA needs to be constructed. Amplified by the diagnostic primers. Optimization of diagnostic primers only. Monitors the functionality of the diagnostic primers specifically. The amount of cIAC is critical to avoid competition. Each assay requires a specific cIAC.</td>
<td>nIAC primers may target any DNA, no need to construct a specific sequence. Amplified by a different set of primers. Optimization of diagnostic primers and nIAC primers. Monitors the functionality of the diagnostic primers by a different set of primers. The amount of nIAC and the amount of nIAC primers can be regulated to avoid competition. One nIAC can be used in different PCR assays.</td>
</tr>
</tbody>
</table>

3.5 Sequencing techniques

As a second step in the diagnostic procedure, the genotype of the parasite may be identified. Genotyping may increase our knowledge about how the degree of virulence of the parasite is connected to its genotype when different hosts are infected. Genotyping is also important in epidemiological studies. In the routine laboratory, analysis of single nucleotide polymorphisms (SNPs) by suitable sequencing techniques can be used to identify *T. gondii* genotypes.

3.5.1 Sanger sequencing

Technologies to determine DNA sequences were invented in the 1970s by Maxam-Gilbert, and by Sanger (Maxam and Gilbert 1977; Sanger, Nicklen *et al.* 1977). Sanger sequencing is the most commonly used technique. The technique is performed by DNA synthesis with deoxynucleotide-triphosphatases (dNTPs),
and labeled dideoxynucleotide-triphosphatases (ddNTPs). Random incorporation of ddNTP terminates the DNA synthesis and results in labeled DNA fragments of different size. The exact DNA sequence can be determined by size separation and analysis of the ddNTP label.

### 3.5.2 Pyrosequencing

The principles of Pyrosequencing were first described in 1985 (Fig 8) (Ronaghi, Uhlen et al. 1998; Ahmadian, Ehn et al. 2006). Pyrosequencing is a sequencing by synthesis method, which is suitable for SNP analysis and microbial typing (Ronaghi 2001; Unnerstad, Ericsson et al. 2001; Ahmadian, Ehn et al. 2006).

**Fig 8:** Pyrosequencing is performed by addition of nucleotides to the reaction mixture, one type of nucleotide (A, T, C or G) at the time. DNA polymerase (oval) incorporates complementary nucleotides, and pyrophosphate (PPI) is released. A quantitative conversion of PPI to ATP by sulfurylase (hexagon) occurs. In the subsequent reaction visible light is produced by firefly luciferase (pentagon), which is quantified and registered as peaks in a Pyrogram. Apyrase (octagon) degrades excess or unincorporated nucleotides before the next set of nucleotides is added.
The method is rapid with a high throughput and can be automated. The technique does not require labeled nucleotides or gel-electrophoresis. The use of the technology is, however, currently restricted to analysis of short DNA sequences. The result of Pyrosequencing is a Pyrogram, which is exemplified in Fig 9.

**Fig 9:** Example of a Pyrogram. The order of added nucleotides is presented on the x-axis, and the intensity of the produced light on the y-axis. Pyrosequencing is initiated by addition of enzyme (E), substrate (S) and a negative control (T). Nucleotides are to the reaction mixture according to a specific dispersion order. Sequencing is performed in real-time, and incorporation of a complementary base results in a so-called "single peak". "Double peaks" have greater amplitude, and corresponds to incorporation of two consecutive bases of the same kind.
4 AIMS

The overall aim of the thesis was to improve the molecular diagnosis of toxoplasmosis in immunocompromised patients.

The specific aims of the thesis were to:

- compare the detection limits of PCR when manual and automated DNA extraction methods were used;
- investigate if repetitive DNA elements increase real-time PCR performance;
- construct and test the use of internal amplification controls;
- develop a rapid molecular method to discriminate between type I, II and III *T. gondii* in clinical samples;
- investigate the magnitude of toxoplasmosis after BMT, using PCR and real-time PCR as diagnostic tools to analyze blood samples;
- investigate the magnitude of pulmonary toxoplasmosis in HIV positive patients, using real-time PCR as diagnostic tool to analyze BAL samples;
- investigate if clinical signs and laboratory data could be identified that would facilitate molecular diagnosis of toxoplasmosis in immunocompromised BMT recipients and HIV positive patients.
5 MATERIALS AND METHODS

The main methods and experimental setups used in the thesis are described below. Detailed information can be found in the papers I-V.

5.1 T. gondii isolates (paper I-V)

The T. gondii tachyzoites (strain RH) used in paper I, and as positive controls in paper IV and V, were from the RH strain, and were provided by Pål A. Jenum, Rikshospitalet, Oslo, Norway. The three strains used in Paper II (RH, Pru and NED), and the 21 strains used in paper III were provided by the BRC ToxoBS group (French parasitologist network for Toxoplasma isolate collection). These isolates had been characterized previously by multilocus analysis based on microsatellite and isoenzyme markers (Darde 1996; Ajzenberg, Banuls et al. 2002).

5.2 Blood samples (paper I-IV)

The spiked blood samples used in paper I were prepared by mixing whole blood with different concentrations of T. gondii tachyzoites of the RH strain. The concentration of the tachyzoites was determined by counting in a Bürker chamber. In paper II, III, and IV, informed consent was obtained from all patients before inclusion in the studies (ethical approval Dnr 91/01, Karolinska University Hospital, Huddinge, Stockholm, Sweden).

5.3 Bronchoalveolar lavage samples (paper V)

Bronchoalveolar lavage (BAL) samples from HIV-positive patients in the Hvidovre HIV cohort attending the Copenhagen University Hospital were used (ethical approval, 2005/65-31/2, Karolinska University Hospital, Huddinge, Stockholm, Sweden).

5.4 DNA extraction (paper I-V)

DNA extraction was performed using two different methods; an automated MagNa pure LC extraction robot (Roche Diagnostics), and a manual QIAamp DNA mini Kit (QIAGEN Inc, Valencia, CA, USA). A MagnaNa pure LC robot was used to extract DNA from T. gondii tachyzoites, T. gondii tissue cysts contained in mouse brain tissue, blood samples, blood samples spiked with
different concentrations of tachyzoites, lung tissue and bronchoalveolar lavage (BAL) samples used in paper I, II, III, and V. A QIAamp DNA mini Kit was used to extract DNA from the 21 *T. gondii* strains and blood samples used in paper I, III and IV. The concentration and quality of the extracted DNA were determined using a Nanodrop® ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) in paper II, III, and IV. The amount of 80 fg DNA was used for each *T. gondii* genome equivalent in paper II and III (Reischl, Bretagne et al. 2003).

5.5 Oligonucleotides (papers I-V)

Oligonucleotides for PCR, real-time PCR, sequencing, and Pyrosequencing were designed using Primer3 (http://fokker.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), Beacon designer (PREMIER Biosoft International, Palo Alto, CA, USA), and by TIB Molbiol (TIB Molbiol, Berlin, Germany). A list of all oligonucleotides used for PCR and sequencing assays is presented in Table 2.

5.6 Real-time PCR analysis of tachyzoites (paper I)

In paper I, it was investigated if the choice of DNA extraction method influences the sensitivity of the PCR. DNA from tachyzoites was extracted using a QIAamp DNA mini Kit and a MagNa pure LC robot. Ten-fold serial dilutions of the DNA extracts were made, and analyzed by LightCycler PCR SYBR green (Table 2). The detection limit of the real-time PCR assay was determined using DNA extracted by the two methods.

5.7 PCR analysis of spiked blood samples (paper I)

In paper I, it was investigated if the choice of DNA extraction method influences the ability of the PCR to detect *T. gondii* in blood samples. Whole blood samples were spiked with different concentration of *T. gondii* tachyzoites, and DNA was extracted using a QIAamp DNA mini Kit and a MagNa pure LC robot. The DNA extracts were analysed using four PCR assays: conventional PCR, nested PCR oligochromatography, real-time PCR SYBR green, and real-time PCR TaqMan (Table 2).
Table 2: Primers and probes used in paper I-V. Oligonucleotide sequences are given 5” to 3”. The TaqMan probes are labeled with FAM or HEX as reporter molecules, and BHQ-1 as quencher molecules.

<table>
<thead>
<tr>
<th>Assay, detection format, and paper</th>
<th>Oligonucleotide, sequence (forward primer, reverse primer, and probe)</th>
<th>DNA target, product length</th>
</tr>
</thead>
</table>
| Conventional PCR, gel analysis, paper I and IV | AAA AAT GTG GGA ATG AAA GAG  
ACG AAT CAA CGG AAC TGT AAT  
ACC ACC AAC TTC ATC CAC GTT CAC C  
CTT CTG ACA CAA CTA GTG TCA TCA GC | $B_1$, 469 bp |
| Nested PCR, oligochromatography, paper I | Sequences not available | $B_1$, not available |
| Real-time PCR, SYBR green, paper I | CTC CTT CGT CCG TCG TAA TAT C  
TGG TGT ACT GCG AAA ATG AAT C | $B_1$, 451 bp |
| Real-time PCR, TaqMan, paper I | TGG TGT ACT GCG AAA ATG AAT C  
CCC CAC AAG ACG GCT GA  
FAM-CAT TGG CAA AAC AGC GGC AGC GTC T-DQ  
TCA CTA GCA ACC TCA AAG AGA  
TGT CTT GTA ACC TTAG ACA AAG  
FAM-CCT CAC CAA CTT CAT CCA CCGT-BHQ-1 | $B_1$, 248 bp |
| Toxo-B1, SYBR green, paper II | CGT CCG TCG TAA TAT CAG  
GAC TTC ATG GGA CGA TAT G | $B_1$, 98 bp |
| Toxo-529, SYBR green paper II | CAC AGA AGG GAC AGA AGT  
TCG CCT TCA TCT ACA GTC | 529 bp repeat element, 94 bp |
| Toxo-529 cIAC, TaqMan, paper II, IV, and V | CAC AGA AGG GAC AGA AGT  
TCG CCT TCA TCT ACA GTC  
FAM-CTC TCC TCC AAC AGC GCT GG-BHQQ1  
HEX-TAA GCC ATC ACA AGC ATC TCA GC-BHQQ1 | 529 bp repeat element, 94 bp |
| Toxo-529 nIAC, TaqMan, paper II | CAC AGA AGG GAC AGA AGT  
TCG CCT TCA TCT ACA GTC  
FAM-CTC TCC TCC AAC AGC GCT GG-BHQQ1  
HEX-TAA GCC ATC ACA AGC ATC TCA GC-BHQQ1  
ATA ACA GGC AAT CCA TAT GA  
ATG AAG TCT ATG GAG AAA CC  
HEX-TAA GCC ATC ACA AGC ATC TCA GC-BHQQ1 | Internal amplification control plasmid, 114 bp |
| GRA6 sequencing, gel analysis, paper V | TGT GGT GTT GGC AGT ATC TGT  
CCC CTG TTT TCA TCT GTA ATA TCT | GRA6 |
| GRA6 Pyrosequencing, SYBR green | Biotin-TGC TCA ATC GTT GGG TGG A  
TGT ATC ATC TCC AGG TAA CGA GTC  
FAM-CAC GCG CCA TCG CCG AAG GTT G-BHQ1  
TCG ACT CAG AAG ATC TGG  
ATG GGC TAT GAA GTA ATG AC  
HEX-TGC GCG ATAG TTA AAG GACGATATC-BHQ1  
CGC AGC AGA CAG CG | GRA6, 176 bp |

5.8 A 529 bp element and the $B_1$ gene as PCR targets (paper II)

To investigate if the detection limit of real-time PCR is improved when a more repeated DNA target is amplified, two similar real-time PCR SYBR green assays were created; one targeting a 200-300-fold repeated 529 bp element, and the other targeting the 35-fold repeated $B_1$ gene (Table 2) (Grigg and Boothroyd...
Molecular diagnosis of infection with Toxoplasma gondii in the immunocompromised patient

2001). The assay targeting the 529 bp repeat element was denoted as “toxo-529”, and the assay targeting the B1 gene as “toxo-B1”. The amplification efficiency and the error of toxo-529 and toxo-B1 were determined by analysis of standard curves. Detection limits and accuracies of toxo-529 and toxo-B1 were investigated when the levels of T. gondii DNA were low in a reaction volume, ten genome equivalents or less.

5.9 Design of IAC (paper II and III)

To construct the cIAC used in paper II, a 130-base oligonucleotide was designed to contain the toxo-529 sense and antisense primer binding sites, but with a probe-binding site that differed from that of the 529 bp repeat element. The oligonucleotide was made double stranded by PCR. The PCR product was cloned into a pcDNA3 plasmid, and the plasmid with the cIAC target was transformed to XL1-blue bacteria. The plasmid was purified from the bacteria after culturing, and sequenced in order to verify that the oligonucleotide had been inserted. In paper II, a set of primers was designed to amplify a fragment of Arabidopsis thaliana DNA. A different set of primers was used in a similar way to amplify a fragment of a pcDNA3 plasmid, used for nIAC in paper III.

5.10 Real-time PCR TaqMan with cIAC or cIAC

TaqMan probes and the toxo-529 primers were used to investigate the amplification efficiency when two different types of internal amplification controls were used; a cIAC and a nIAC. The TaqMan assay including the cIAC was denoted ”toxo-529-cIAC”, and the TaqMan assay including the nIAC as ”toxo-529-nIAC” (Table 2). The cIAC plasmid was used as IAC in toxo-529-cIAC, in an amount such that the CP was around 35. A. thaliana DNA was used as nIAC in toxo-529-nIAC, and amplified by a different set of primers, Table 2. The amount of A. thaliana DNA was chosen such that the CP was around 35. Amplification efficiencies and errors of toxo-529-cIAC and toxo-529-nIAC were determined. One µg of human blood cell DNA was included in each reaction volume. One T. gondii genome equivalent in a reaction volume was analyzed by toxo-529-cIAC and by toxo-529, and the results were compared.

5.11 Toxo-529-cIAC

Specificity of toxo-529-cIAC was tested using three strains of T. gondii, RH (type I), NED (type II), and Prugniad (type III) (paper II). The procedure was also tested with 352 blood samples from immunocompromised patients, and the results were compared with results obtained using a conventional PCR assay.
(paper IV) (Jalal, Nord et al. 2004) In paper II, the use of the cIAC to monitor of inhibition caused by non-specific DNA was investigated. Samples containing one *T. gondii* genome equivalent and samples negative for *T. gondii* were analyzed by toxo-529-cIAC in the presence of different amounts of non-specific DNA. Reproducible amplification of a balanced cIAC was tested by analysis of 50 blood samples from BMT recipients.

5.12 Pyrosequencing (paper III)

Primers and probes for a real-time PCR assay targeting the *GRA6* gene were designed, and used to amplify sequence templates for Pyrosequencing (Table 2). A pcDNA3 plasmid was used as IAC and amplified by a set of non-biotinylated primers. As described in paper II, a minimal detectable amount of IAC was used in a reaction volume. Amplification efficiency and error of the real-time PCR assay were determined. Discrimination of type I, II and III *T. gondii* was performed by Pyrosequencing analysis of two SNPs, located at the previously described positions 162 and 171 of the *GRA6* gene (Fazaeli, Carter et al. 2000). The corresponding SNPs for type II were G and G, and for type III A and A. Reproducibility (R), and typeability (T) of the Pyrosequencing assay were determined by three independent analyses of 21 *T. gondii* strains. R was calculated according to the following equation: \( R = \frac{N_r}{N} \) (\( N_r \) = the number of isolates assigned the same type on repeat testing, and \( N \) = the number of isolates tested). T, which is a measure of the ability of the Pyrosequencing assay to discriminate between the different genotypes tested, was calculated according to the following equation: \( T = \frac{N_t}{N} \) (\( N_t \) = the number of typeable strains, and \( N \) = the number of isolates tested). The methods ability to detect mixtures of type I, II and III in a reaction volume was tested.

5.13 Clinical applications of toxo-529-cIAC and Pyrosequencing

The clinical utility of toxo-529-cIAC and Pyrosequencing was demonstrated by monitoring one case of toxoplasmosis after allogeneic BMT, using peripheral blood samples drawn at three consecutive days, and lung tissue taken at autopsy, paper II and III. In paper IV, toxo-529-cIAC was used to analyze peripheral blood samples from BMT recipients, and in paper V, toxo-529-cIAC was used to analyze BAL samples from HIV-positive immunocompromised patients.
5.14 Prospective study of toxoplasmosis after BMT (paper IV)

Thirty-three BMT recipients were monitored prospectively by PCR analysis of peripheral blood samples to investigate the magnitude of toxoplasmosis (Table 3). Toxo-529-cIAC and conventional PCR, described in paper I and II, were used. Seven of the 21 autologous transplant recipients (9 females and 14 males, mean age 55 years), and 4 of the 12 allogeneic transplant recipients (4 females and 8 males, mean age 43 years) were seropositive for *T. gondii*. In total, 352 blood samples were collected during the study period. The number of samples per patient 1 week before and 1-3 months after BMT were 2-14, for 4-6 months 0-6, and for 7-12 months 0-1. Medical records were analyzed using a standardized form to identify risk factors and clinical symptoms of toxoplasmosis. All patients received *Pneumocystis jirovecii* prophylaxis, consisting of TMP/SMX (Ringden, Remberger et al. 1995).

5.15 Pulmonary toxoplasmosis in HIV patients (paper IV)

Three hundred and thirty-two BAL samples from 290 HIV-positive patients were taken after unexplained pulmonary symptoms. All patients suffered from advanced HIV disease. Extracted DNA was analyzed using toxo-529-cIAC (paper II). Medical records were retrieved for the patients whose BAL samples contained *T. gondii* DNA. A fragment of the *GRA6* gene was amplified and sequenced to discriminate between type I, II and III *T. gondii* by analysis of previously described single nucleotide polymorphisms (SNPs) in the *GRA6* gene (Table 2) (Fazaeli, Carter et al. 2000).
Table 3: Twenty-one patients were treated with autologous BMT (auto), and 12 with allogeneic BMT (allo). Anti-toxoplasma IgM and IgG antibodies are measured in international units. M = male, F = female, ALL = acute lymphoid leukemia, AML = acute myeloid leukemia, CML = chronic myeloid leukemia, CyA = cyclosporin, MTX = methotrexate, MMF = mucophenolate motilf, MUD = matched unrelated donor, PBSC = peripheral blood stem cell, ATG = antithymocyte globulin, fTBI = fractionated total body irradiation, Cy = cyclophosphamide, Bu = busulphane, Flu = fludarabin.

<table>
<thead>
<tr>
<th>Sex and age</th>
<th>Serology</th>
<th>Underlying disease</th>
<th>Immuno-suppression</th>
<th>Transplantation type</th>
<th>Conditioning</th>
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<tr>
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<td>bu + cy</td>
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<tr>
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<tr>
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<tr>
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</tr>
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<td>Auto</td>
<td>BEAM</td>
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<td>Auto</td>
<td>BEAM</td>
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<tr>
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<td>Carbo + VP-16 + cyclofosfamid</td>
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<tr>
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<td></td>
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<td>Myeloma</td>
<td>Auto</td>
<td>Melphalan 200 mg/m²</td>
<td></td>
</tr>
<tr>
<td>M 66</td>
<td>Negative</td>
<td>Myeloma</td>
<td>Auto</td>
<td>Melphalan 100 mg/m²</td>
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</tr>
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<td>CyA + MTX</td>
<td>Allo, MUD, PBSC</td>
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<tr>
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<td>CyA + MMF</td>
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<tr>
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<td>CyA + MMF</td>
<td>Allo, MUD, bone marrow</td>
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<tr>
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<td>AML</td>
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<td>Allo, MUD, PBSC</td>
<td></td>
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<tr>
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<td>KML</td>
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<td>Allo, MUD, PBSC</td>
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<tr>
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<td>AML</td>
<td>CyA + MTX</td>
<td>Allo HLA sibling, PBSC</td>
<td></td>
</tr>
<tr>
<td>F 56</td>
<td>IgG 7.0</td>
<td>AML</td>
<td>CyA + MTX</td>
<td>Allo MUD, PBSC</td>
<td></td>
</tr>
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<td>AML</td>
<td>CyA + MTX</td>
<td>Allo MUD, PBSC</td>
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<tr>
<td>M 52</td>
<td>Negative</td>
<td>AML</td>
<td>CyA + MTX</td>
<td>Allo MUD, PBSC</td>
<td></td>
</tr>
<tr>
<td>M 26</td>
<td>Negative</td>
<td>CML</td>
<td>CyA + MTX</td>
<td>Allo MUD, bone marrow</td>
<td></td>
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<tr>
<td>F39</td>
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<td>CyA + MMF</td>
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<tr>
<td>M 54</td>
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<td>ALL</td>
<td>CyA + MTX</td>
<td>Allo MUD, bone marrow</td>
<td></td>
</tr>
</tbody>
</table>

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6 RESULTS AND DISCUSSION

6.1 Comparison of manual and automated DNA extraction

DNA extraction is one of the important steps to successfully detect the small amounts of *T. gondii* tachyzoites that may be expected in clinical samples. It is therefore important to investigate if the choice of DNA extraction method influences the detection limit of the subsequent PCR assay. In a first step, we compared the detection limits of a real-time PCR SYBR green assay targeting the *B1* gene, when DNA from pure *T. gondii* tachyzoites had been extracted manually using a QIAamp DNA mini Kit, and using an automated system, a MagNa pure LC robot. The detection limit of the real-time PCR assay was the same using the two DNA extraction methods; one *T. gondii* genome equivalent in a reaction volume was detected. Thus the results presented in paper I show that the two DNA extraction methods work with equal efficiency for extraction of DNA from *T. gondii* tachyzoites alone (Fig 10).

![Graph showing mean crossing points (CP) for three different ten-fold serial dilutions of *T. gondii* DNA, extracted using a MagNa pure LC robot or a QIAamp DNA mini Kit.]

Fig 10: Mean crossing points (CP) for three different ten-fold serial dilutions of *T. gondii* DNA, extracted using a MagNa pure LC robot or a QIAamp DNA mini Kit.
In a second step, we investigated if the detection limits of four PCR assays (all with a detection limit of one parasite genome in a reaction volume when analyzing *T. gondii* DNA alone) varied depending on extraction method when different amounts of pure *T. gondii* tachyzoites were mixed with whole blood. It was clear that SYBR green chemistry could not be used to analyze whole blood samples, probably due to unspecific incorporation of SYBR green molecules in blood cell ds-DNA with a high background signal as a result (Table 4) (Bellete, Flori *et al.* 2003). Real-time PCR TaqMan had a detection limit of $10^2$ tachyzoites in a reaction volume, which was a 100-fold decrease compared to a situation when pure *T. gondii* tachyzoites were analyzed. It was later shown in paper II that a decreased performance of real-time PCR TaqMan might be expected when exceeding certain amounts of non-specific DNA in a reaction volume. This may explain the reduced sensitivity of the TaqMan assay in paper I.

Concerning non-specific DNA, it was demonstrated in paper IV that DNA extraction of a fixed volume of whole blood from BMT recipients may result in a great variation of the DNA concentrations. Indeed, the concentrations of the DNA extracts of 352 blood samples from BMT recipients ranged from 4 to 917 ng/µl, with a median value of 212 ng/µl. The results in paper I, II and IV taken together suggest that probes are the preferred LightCycler PCR chemistry to detect *T. gondii* in whole blood samples. It should be considered that reduced amplification efficiency, as a result of inhibition when analyzing whole blood samples, might cause a standard curve used for quantitative analysis of parasites to be less reliable. We recommend that i) the standards used to construct a standard curve should include an equal amount of total DNA per reaction volume that will be used when unknown samples are analyzed, ii) that DNA is quantified prior to real-time PCR analysis, and iii) that an internal amplification control is used to detect inhibition in *T. gondii* negative samples (paper II).

The detection limits of nested PCR oligochromatography and real-time PCR TaqMan were the same regardless of the extraction method (Table 4). Conventional PCR, on the other hand, was more sensitive when DNA was extracted using the QIAamp DNA mini Kit, and interestingly, the sensitivity was reduced when the MagNa pure LC system was used. The potential presence of assay-specific PCR inhibitors when *T. gondii* DNA is amplified by different set of primers has been discussed in a previous study, where it was shown that PCR using one set of primers might be inhibited while PCR performed with a different set of primers is not (Filisetti, Gorci *et al.* 2003). An alternative explanation might be attributed to the gel analysis which is a required step when conventional PCR is performed. Discrimination of a dim positive band from a
negative result can be difficult. Oligochromatography and real-time PCR, on the other hand, generated clear results.

Interestingly, it was also found that a conventional, single step PCR assay was at one occasion more sensitive than a nested PCR protocol. Hence, a carefully designed and properly optimized single round of PCR may be sufficient to achieve the same level of sensitivity as when a nested PCR protocol is used, presupposed that the quality of the extracted DNA is sufficient. A nested PCR system may however increase the sensitivity due to its dilution effect between the first and the second round of PCR if inhibitory factors are present. There is, however, a risk for carry-over contamination between a first and a second round of PCR, and thereby false positive results which may make a nested approach less suitable in the routine laboratory.

Table 4: Top: Comparison of detection limits of conventional, nested and real-time PCR assays when DNA from spiked blood samples was extracted using a MagNa pure LC robot. Each method was tested with two samples, each sample was analyzed in duplicate where + indicates a positive PCR, and – indicates a negative PCR. * = number of tachyzoites used for spiking of 2 ml EDTA blood. One tenth of the extracted DNA was included in a PCR sample, and the results presented here correspond to the methods ability to detect *Toxoplasma gondii* in 2 ml blood, and not in a PCR reaction volume. Bottom: Comparison of detection limits of conventional, nested and real-time PCR assays when DNA from spiked blood samples was extracted using a QIAamp DNA minikit.
6.2 Repeated DNA elements and real-time PCR performance

A variety of real-time PCR assays have been developed for rapid and sensitive detection of *T. gondii* DNA. Different PCR targets have also been tested. It has been suggested that a recently described 529 bp element of unknown function, which is repeated 200-300 times in the genome of *T. gondii*, would increase the analytical sensitivity of the PCR to a level greater than that obtained when targeting the less repeated *B1* gene (Homan, Vercammen *et al.* 2000; Filisetti, Gorcii *et al.* 2003; Reischl, Bretagne *et al.* 2003; Montoya and Liesenfeld 2004; Remington, Thulliez *et al.* 2004). However, the sensitivity and accuracy of using the 529 bp element must be further evaluated, particularly in samples in which the level of *T. gondii* DNA is low. In paper II, a study was designed in which 10 *T. gondii* genome equivalents or less in a reaction volume were analyzed. The two similarly designed real-time PCR assays that were used, toxo-B1 and toxo-529, enabled us to compare the sensitivity and accuracy of real-time PCR targeting the 529 bp repeat element with the results obtained when targeting the *B1* gene.

<table>
<thead>
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<th>DNA (fg)</th>
<th>Toxo-529</th>
<th>Toxo-B1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>positive</td>
<td>mean CP</td>
</tr>
<tr>
<td>800</td>
<td>30</td>
<td>28.9</td>
</tr>
<tr>
<td>80</td>
<td>30</td>
<td>32.3</td>
</tr>
<tr>
<td>8</td>
<td>10</td>
<td>36.1</td>
</tr>
<tr>
<td>0.8</td>
<td>7</td>
<td>38.0</td>
</tr>
</tbody>
</table>

The amplification efficiency of toxo-529 was 1.95 and the error was 0.036, while the amplification efficiency of toxo-B1 was 1.91 and the error was 0.081. The performances of the two assays were considered as satisfactory. The detection limit was defined as the amount of *T. gondii* DNA in a reaction volume for which all analyzed samples gave a positive result. The detection limit of toxo-529 was one genome equivalent (80 fg DNA) in a sample, while that of toxo-*B1* was 10 parasite genomes (800 fg DNA) in a sample (Table 6). Hence, toxo-529 is more sensitive than toxo-B1. Further, the coefficient of
variation (C.V.) was lower when *T. gondii* DNA was analyzed with toxo-529 than with toxo-B1. The results presented in paper II suggest that real-time PCR targeting a more repeated DNA element performs better in terms of quantitative accuracy and detection limit.

### 6.3 Real-time PCR including IAC

Internal amplification control systems are important in order to detect whether the PCR has been inhibited. In some cases these controls may, however, reduce the sensitivity and accuracy of the diagnostic PCR. This may be caused by an undesirable competition between the efficiency with which the *T. gondii* DNA is amplified, and the efficiency with which the IAC is amplified (Hoorfar, Malorny et al. 2004). In paper II, it was investigated whether an IAC can be used in real-time PCR targeting the 200-300-fold repeated 529 bp element for the diagnosis of toxoplasmosis without degrading the diagnostic performance of the PCR. Amplification efficiency and error of toxo-529-cIAC and toxo-529-nIAC were compared. The amplification efficiency of toxo-529-cIAC was 1.90 and the error 0.041, while the equivalent figures for toxo-529-nIAC were 1.90 and 0.003. The results show that amplification of the 200-300-fold repeated 529 bp element can be performed without affecting the amplification efficiency when a cIAC or a nIAC is included. As discussed in paper I, inhibitory factors may affect one set of primers while a different set of primers is not affected. Thus the use of a cIAC may be considered as a more specific control system as it depends directly on the function of the diagnostic set of primers. In addition, the use of a cIAC is more convenient as it eliminates the need for an additional set of primers. The cIAC was therefore chosen for the subsequent experiments.

The results presented in paper II also show that the sensitivity and accuracy of real-time PCR targeting the 200-300-fold repeated 529 bp element are not affected by the presence of cIAC, and 1 µg of human blood cell DNA when analyzing one *T. gondii* genome equivalent in a reaction volume. Thirty PCR samples containing 80 fg *T. gondii* DNA were positive when analyzed with toxo-529-cIAC and toxo-529. The mean crossing point for toxo-529-cIAC was 33.6 and its coefficient of variation (C.V.) was 2.2%. The equivalent figures for toxo-529 (performed without inclusion of human blood cell DNA and IAC) were 32.3 and 2.8%.

Two strategies were used to keep the amplification of the cIAC from competing with that of *T. gondii* DNA. Firstly, the cIAC was balanced to a minimal but reproducibly detectable concentration, as demonstrated in paper II and III. In paper II, a balanced amount of cIAC was amplified when 50 blood samples from BMT recipients, all negative for *T. gondii*, were tested. The mean CP was
35.4, and C.V. was 2.2%. This shows that the amount of IAC could be balanced to a small but reproducibly detectable amount in a reaction volume which requires more PCR cycles to generate a detection signal than the lowest detectable amount of *T. gondii* DNA does, which had a CP of 33.6. In this way balancing of the cIAC could be utilized to avoid undesirable competition when low amounts of *T. gondii* DNA in a reaction volume (1 genome equivalent) are analysed. Secondly, the amplification efficiency was around 1.5-1.6 for the IACs used in toxo-529-cIAC, toxo-529-nIAC, and real-time PCR targeting the *GRA6* gene (data not shown for 529-cIAC, toxo-529-nIAC). In theory, a PCR with lower amplification efficiency should be outcompeted if the starting amounts of target DNA are equal during co-amplification.

### 6.4 Monitoring of PCR inhibition by a cIAC

High amounts of non-specific DNA in a sample inhibit amplification of the *B1* gene when performed using a LightCycler instrument (Bellete, Flori *et al.* 2003). **Paper II** shows that this is also the case when a more repeated DNA element is amplified, the 200-300-fold repeated 529 bp element, and that a cIAC can be used to monitor inhibition of the PCR.

![Fig 11](image.png)

**Fig 11:** Influence of different amounts of human blood cell DNA on the performance of real-time PCR, including a competitive internal amplification control (cIAC). One *T. gondii* genome equivalent (80 fg of DNA) was mixed with 1, 1.5, 2 or 4 µg of human blood cell DNA. Samples without *T. gondii* DNA were also analyzed. Toxo-529 shows the crossing points (CP) for *T. gondii* DNA amplification, and cIAC shows CPs for the cIAC in samples without *T. gondii* DNA. The CP increases with increased amounts of non-specific DNA, which shows that the PCR is inhibited. The cIAC is sensitive for inhibition and can be used to detect inhibition in *T. gondii* negative samples. When samples containing 4 µg of non-specific DNA are analyzed, inhibition of the PCR is so severe that the cIAC is not amplified.
Experiments performed with one *T. gondii* genome equivalent in a reaction volume showed that the CP at which the *T. gondii* DNA was detected increased when levels of non-specific DNA greater than 1 µg were present, as did the crossing point of cIAC (Fig 11). Thus, cIAC amplification is inhibited in the same manner as amplification of *T. gondii* DNA. We conclude that a cIAC can be used to reveal inhibition of the PCR if *T. gondii* DNA is not amplified. **Paper II and IV** demonstrates how overload of sample DNA can be avoided by the use of a NanoDrop® ND-1000 Spectrophotometer. The quantity and quality of extracted DNA can be determined rapidly and accurately, using no more than 1 µl of sample material.

### 6.5 Specificity of toxo-529-cIAC

In **paper IV**, 352 blood samples from BMT patients were analyzed using toxo-529-cIAC. The results were compared to those obtained with the conventional PCR assay targeting the *B1* gene, used in **paper I**. *T. gondii* DNA was found in one blood sample by the two methods, the remaining 351 were negative. Inhibition of the PCR was not detected. Positive PCR signals were obtained when toxo-529-cIAC was tested with DNA from type I, II and III *T. gondii* (**paper II**).

### 6.6 Discrimination of *T. gondii* genotypes by Pyrosequencing

The clinical consequences of toxoplasmosis depend on host characteristics, but also on the genotype of the infecting strain (Howe and Sibley 1995; Howe, Honore et al. 1997; Grigg, Ganatra et al. 2001; Khan, Su et al. 2005). To further elucidate the significance of the parasite genotype in human toxoplasmosis, genotyping needs to be performed to a larger extent in clinical findings. A second part of the molecular diagnosis may therefore include genotyping of the parasite. Rapid and user-friendly molecular techniques are needed for this purpose.

**Paper III** demonstrates how real-time PCR and Pyrosequencing can be used to rapidly discriminate between type I, II and III *T. gondii* strains. The technique is far more rapid and convenient to use than the Sanger sequencing technique, which was used in **paper V**. Discrimination of type I, II and III was performed by Pyrosequencing analysis of a 10-base fragment of the *GRA6* gene, including two SNPs specific for type I, II and III (Fig 12). Type I, II, and III isolates were correctly identified according to previous typing results, while the four atypical isolates were identified as *GRA6* type I or III when the 21 isolates were analyzed.
(Table 7). The reproducibility of the Pyrosequencing assay was 100%, and the typeability was 81%.

![Pyrosequencing analysis of type I, II, and III T. gondii](image)

Fig 12: Pyrosequencing analysis of type I, II, and III T. gondii. The previously described single nucleotide polymorphisms (SNPs) in the GRA6 gene at positions 161, corresponding to peaks 2 and 3, and 172, correspond to peaks 9,11, were analyzed. A) GRA6 type I, SNPs were G and A. B) GRA6 type II, SNPs were G and G. C) GRA6 type III, SNPs were A and A. D) Pyrosequencing analysis of mixture of type I and II. Discrimination of mixtures of two different GRA6 types required analysis of presence or absence of ”single peaks”, and analysis of ”single peaks” with increasing amplitudes towards ”double peaks”, or the opposite direction. The first SNP is G, while the second is both A and G, indicating a mixture of GRA6 type I and II.

Sequencing of the GRA6 gene has revealed numerous SNPs (Fazaeli, Carter et al. 2000). Most likely, analysis of several independent genetic markers is required if the aim is to identify atypical strains (Darde 2004; Ajzenberg,
Dumetre et al. 2005). A challenge is that the read-length of Pyrosequencing is limited, and genetic markers suitable for Pyrosequencing must therefore provide sufficient discriminatory power by analysis of short DNA sequences. In paper III, it was shown that Pyrosequencing could be used to discriminate between two different genotypes in a sample (Fig 12). The results were, however, hard to interpret when the genotypes were present in different amounts. This part of the assay needs to be further developed.

Table 7: Pyrosequencing of the *GRA6* gene performed using 21 isolates of *T. gondii*. The results obtained when using Pyrosequencing to characterize type I, II or III isolates were in agreement with previous characterizations performed by analysis of five microsatellite markers and five isoenzyme markers. Pyrosequencing identified atypical strains as *GRA6* type I or III.

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<th>Strain</th>
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<th>Previously characterized as</th>
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<tbody>
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<td>I</td>
<td>I</td>
</tr>
<tr>
<td>ENT</td>
<td>I</td>
<td>I</td>
</tr>
<tr>
<td>GIL</td>
<td>I</td>
<td>I</td>
</tr>
<tr>
<td>CTI</td>
<td>I</td>
<td>I</td>
</tr>
<tr>
<td>ATIH</td>
<td>I</td>
<td>I</td>
</tr>
<tr>
<td>S3K</td>
<td>II</td>
<td>II</td>
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Real-time PCR targeting the *GRA6* gene is a required step to produce Pyrosequencing templates. This real-time PCR assay was however not as sensitive as the real-time PCR assays described in paper II, where more
repeated PCR targets were utilized. In papers IV and V, this became a problem when *T. gondii* DNA was detected by toxo-529-cIAC, and attempts to perform genotyping by GRA6 analysis failed due to the low amount of *T. gondii* DNA in the sample. To improve the sensitivity and typeability of the Pyrosequencing assay, the search for suitable genetic markers should, if possible, involve repeated DNA targets. Ultimately, a single round of PCR would be performed to detect the parasite with sufficient sensitivity, and in a subsequent reaction determine its genotype by Pyrosequencing.

### 6.7 Application to a clinical case

Real-time PCR and Pyrosequencing were used in a clinical context to diagnose toxoplasmosis after allogeneic BMT (paper II and III). In brief, a 63-year-old female patient, with a clinical record of chronic myelogenous leukemia, and relapse after autologous transplantation, was treated with allogeneic stem cell transplantation. The patient was seropositive for *T. gondii* before transplantation. TMP/SMX prophylaxis was dropped due to skin rash. The patient reported headache, and later developed fever and symptoms of an upper respiratory tract infection including cough. Magnetic resonance imaging of the brain revealed a focal lesion. At day 3 the patient died. The amount of *T. gondii* DNA in peripheral blood samples drawn at 3 consecutive days was quantified using toxo-529-cIAC (Fig 14). A high amount of *T. gondii* DNA was found in lung tissue taken at autopsy.

![Figure 14: Toxo-529-cIAC was used to monitor parasitemia in blood in a transplant recipient suffering from reactivated toxoplasmosis. Treatment for toxoplasmosis was initiated at Day 1. The level of *T. gondii* DNA in blood increased despite treatment and the patient died on Day 3. Each analysis took only 2 hours (including DNA extraction).](image-url)
Molecular diagnosis of infection with Toxoplasma gondii in the immunocompromised patient

Treatment was unable to stop the development of reactivated toxoplasmosis after BMT, and this may be due to delay in installing treatment. The disease had a sudden onset and a rapid course, which lead to the death of the patient. The genotype was found to be GRA6 type II in both blood and lung tissue (paper III). In this particular case, it was difficult to conclude to what extent the genotype of the parasite affected the course of the infection. Type II \textit{T. gondii} has previously been associated with severe cases of human toxoplasmosis (Howe and Sibley 1995; Howe, Honore \textit{et al.} 1997; Fuentes, Rubio \textit{et al.} 2001; Ajzenberg, Cogne \textit{et al.} 2002).

6.8 Molecular diagnosis of toxoplasmosis after BMT

It is not well known how frequently toxoplasmosis occurs after BMT, or if reactivated but asymptomatic toxoplasmosis have a clinical relevance (Bretagne, Costa \textit{et al.} 2000). The magnitude of toxoplasmosis after BMT has rarely been studied prospectively to further evaluate this (Bretagne, Costa \textit{et al.} 2000; Martino, Bretagne \textit{et al.} 2005). A prospective study by PCR analysis of peripheral blood samples from BMT recipients, paper IV, suggests that the risk for toxoplasmosis in BMT recipients is low, with \textit{T. gondii} detected in 1 of 33 patients. However, one of 10 seropositive patients had asymptomatic toxoplasmosis during the time of the study, shown by a low number of \textit{T. gondii} genome equivalents detected in blood by PCR only 5 days post BMT. The number of \textit{T. gondii} genome equivalents was around 10 per ml blood, and there were no clinical signs of toxoplasmosis.

Asymptomatic toxoplasmosis was detected by PCR despite TMP/SMX prophylaxis, intended at \textit{Pneumocystis Jirovecii} but also effective against \textit{T. gondii}. The prophylactic regimen did, however, probably prevent full-blown toxoplasmosis. As mentioned above, it is not well known what clinical significance asymptomatic toxoplasmosis has when shown by positive PCR in peripheral blood. Nevertheless, it is possible that the phenomenon may repeat itself during immunosuppressive therapy, and possibly cause disease later on. The finding suggests that surveillance of allogeneic BMT recipients by PCR should be initiated immediately after transplantation to be effective.

Paper II also shows that an ocular infection of unknown aetiology was observed in an allogeneic BMT recipient, seropositive for \textit{T. gondii}. The patient had a medical history of reactivated cytomegalovirus (CMV) infections, but CMV was not identified as the causative agent of the ocular pathology. It is tempting to speculate that \textit{T. gondii} was involved in this ocular infection.
The results presented in paper IV confirm previous conclusions; focused attention should be given to the seropositive allogeneic transplant recipients, whereas active infections are rarely seen in those seronegative or autologously transplanted (Derouin, Gluckman et al. 1986; Slavin, Meyers et al. 1994; Chandrasekar and Momin 1997; Martino, Maertens et al. 2000; de Medeiros, de Medeiros et al. 2001; Martino, Bretagne et al. 2005). Indeed, our results show that a recent primary infection with T. gondii, indicated by the presence of IgM antibodies, did not result in toxoplasmosis detected by PCR.

A risk profile for toxoplasmosis in BMT recipients would be useful to select patients who would benefit from monitoring by PCR. A review of the medical records did not reveal any clinical signs or laboratory data that could be used to identify BMT recipients who would benefit from such monitoring. The clinical signs usually associated with toxoplasmosis, for example fever, graft-versus-host-disease (GVHD), and other infections, were also observed in patients in whom T. gondii DNA was not detected by PCR. None of these clinical signs were observed when T. gondii DNA was found by PCR in peripheral blood from an allogeneic BMT recipient, and a specific risk profile for toxoplasmosis after BMT could not be identified. The results do however suggest that monitoring of allogeneic BMT recipients by PCR may have to be initiated immediately after transplantation, and that monitoring may have to include all seropositive patients in this group to be meaningful. As shown by us and others, peripheral blood samples seem to be a suitable sample material (Bretagne, Costa et al. 2000; Martino, Bretagne et al. 2005).

### 6.9 Pulmonary toxoplasmosis in HIV-positive patients

Pulmonary toxoplasmosis is a well-known opportunistic infection in immunocompromised patients, and is associated with a high rate of mortality (Evans and Schwartzman 1991; Pomeroy and Filice 1992). As in BMT recipients, diagnosis of toxoplasmosis in severely immunocompromised HIV patients is challenging as the clinical signs may be non-specific.

In paper V, BAL samples, which were taken at the onset of unexplained pulmonary symptoms in HIV-positive patients, were analyzed by real-time PCR to investigate the magnitude of pulmonary infections involving T. gondii. T. gondii DNA, corresponding to one genome equivalent or more, was found in BAL samples from seven patients. Genotyping was possible in two samples, and the parasite was found to be GRA6 type II. Medical records for six of these patients could be retrieved. Clinical data is presented more in detail in paper V. Five of the six patients were seropositive for T. gondii, which is in agreement with previous findings that only a small proportion of immunosuppressed HIV-
positive patients with toxoplasmosis are seronegative (Garly, Petersen et al. 1997).

The six patients had multiple clinical symptoms, but all had pulmonary symptoms of cough or dyspnea. The patient with the highest amount of *T. gondii* DNA in BAL was the only patient receiving oxygen treatment for hypoxia, and it is possible that the parasite load contributed to the reduced pulmonary function. Four patients had pulmonary infections with *P. jirovecii*, diagnosed microscopically. There were no differences in the CD4+ T-cell counts, arterial oxygen partial pressure, or chest radiography when compared to the remaining cohort, and a specific risk profile for pulmonary toxoplasmosis in HIV patients could not be identified.

Two patients with *T. gondii* DNA in BAL had symptoms of ocular toxoplasmosis. CMV retinitis was suspected, but not proven, and the patients received treatment for suspected CMV infection. As postulated in paper IV, ocular infections with possible involvement of *T. gondii* may occur in immunocompromised patients who are seropositive for *T. gondii*.

All patients received TMP/SMX prophylaxis against *P. jirovecii*. The results presented in paper IV and paper V indicate that TMP/SMX prophylaxis may not fully protect the immunocompromised host against toxoplasmosis. According to the results presented in paper V, it is difficult to give any recommendations about how monitoring of immunocompromised HIV patients by PCR should be performed, other than if BAL samples are taken after unexplained pulmonary symptoms, the microbiological analysis should also include a PCR test for *T. gondii*. 
7 CONCLUDING REMARKS

As shown in this thesis, the choice of a manual or an automated DNA extraction method did not influence the detection limit of real-time PCR SYBR green when DNA from *T. gondii* tachyzoites was analyzed. Different results were, however, obtained when *T. gondii* tachyzoites were mixed with whole blood and analyzed by conventional PCR, nested PCR oligochromatography, real-time PCR SYBR green, and real-time PCR TaqMan. The results suggest that detection limits may vary depending on sample material, and the choice of DNA extraction method. Concerning real-time PCR, the results show that probes are the preferred chemistry to detect *T. gondii* DNA in samples with non-specific DNA, for example extracts of whole blood.

The choice of PCR target was also found to affect the performance of real-time PCR. Low concentrations of *T. gondii* DNA in a reaction volume was detected more sensitively, and more accurately, when a more repeated DNA target was used, a 200-300-fold repeated 529 bp element, compared to the use of the 35-fold repeated *B1* gene. Furthermore, the performance of real-time PCR targeting the 529 bp element was not affected when a cIAC was included. A cIAC does not require the use of an additional set of primers, and may be the preferred control system when a repeated PCR target, such as the 529 bp element, is amplified. In the future, the 529 bp repeat element may be the preferred target when real-time PCR is used for diagnosis of toxoplasmosis. However, this PCR target has recently been described, and it must be confirmed that it is conserved between different strains of *T. gondii*.

Genotyping of *T. gondii* in clinical findings needs to be performed in larger series of cases to further evaluate the virulence of different genotypes in human toxoplasmosis. Rapid and user-friendly techniques are needed. The Pyrosequencing assay that was developed to discriminate between *GRA6* type I, II and III *T. gondii* in clinical samples is suitable for routine use, although the results show that analysis of a single genetic marker may not be sufficient to detect atypical strains. To increase the typeability of Pyrosequencing, more genetic markers needs to be identified and tested using the technique. Multiplex approaches where more than one genetic marker is analyzed in a reaction volume may be possible. An alternative approach could be to analyze multiple genetic markers in separate reaction volumes, in an “array-like” Pyrosequencing assay to increase the typeability of the method, although factors such as the number of genetic markers, the number of expected samples, throughput, costs, and ease of use should be considered.
Most likely, automated DNA extraction and real-time PCR technologies will be further developed in terms of rapidness, cost effectiveness, and ease of use. An effect that should be considered is the fact that PCR amplifies parasite DNA from live and dead parasites. Hence, the number of viable parasites in blood may be overestimated depending on how stable *T. gondii* DNA is. An alternative approach would be to develop a rapid molecular method to quantify parasite RNA (Cultrera, Seraceni et al. 2002). It remains to be seen if detection of parasite DNA by real-time PCR will be replaced by detection and quantification of parasite RNA, which unlike analysis of DNA has the potential to discriminate between different life-forms of the parasite, and between dead and live parasites which would more accurately reflect the effect of treatment. Alternatively, detection of parasite DNA may remain as a first step in the diagnostic procedure and for genotyping purposes, and may in the future perhaps be performed without an amplification step needed, for example by sensitive hybridization techniques.

A prospective study showed that it was not possible to identify a specific risk profile associated with an increased risk for toxoplasmosis in BMT recipients. Nevertheless, it seems important that surveillance by PCR is initiated immediately after allogeneic BMT in seropositive patients, and peripheral blood samples seem to be a suitable sample material. A retrospective study of pulmonary toxoplasmosis in immunocompromised HIV patients confirmed that a particular risk profile could not be identified. The conclusion was made that a PCR test for *T. gondii* should be included in the microbiological analysis of BAL samples taken after pulmonary symptoms of unknown aetiology in HIV patients. Our results suggest that toxoplasmosis may be suspected if ocular infections of unknown aetiology occur in seropositive allogeneic BMT recipients and HIV patients, and that asymptomatic and symptomatic toxoplasmosis may occur despite TMP/SMX prophylaxis. In both allogeneic transplant recipients and HIV patients, monitoring of seropositive immunocompromised patients using real-time PCR may serve multiple purposes: i) to diagnose active toxoplasma infections at an early stage to facilitate effective treatment, ii) to correlate the parasitic burden to clinical symptoms, and iii) to evaluate the ability of prophylactic regimens to prevent reactivations and full-blown toxoplasmosis. Larger prospective studies are needed to further evaluate if a specific risk profile for toxoplasmosis in seropositive immunocompromised patients can be established.
8 POPULÄRVETENSKAPLIG SAMMANFATTNING

Den encelliga parasiten Toxoplasma gondii är vanligt förekommande i hela världen. Hos människa kan en första infektion vara helt utan symptom, varefter parasiten övergår i en vilande cystform som kan återfinnas i t.ex. hjärna eller muskelvävnad. En vilande infektion är vanligtvis helt utan komplikationer. I Stockholmsområdet har ca 14 % av gravida kvinnor T. gondii i vilande form, medan förekomsten i Skåne är ca 26 %. Människor infekteras vanligen via munnen genom infektiösa oocystor som sprids via kattens avföring eller genom att förtära otillräckligt upphettat kött som innehåller parasiten.


Diagnostik av toxoplasmos hos patienter med nedsatt immunförvar görs genom att påvisa en del av parasitens arvsmassa, dess DNA, med polymerase chain reaction (PCR). En mängd olika PCR metoder har utvecklats för detta ändamål. I denna avhandling visar vi att PCR-resultaten kan variera beroende på typ av PCR metod och typ av provmaterial. Det är därför mycket viktigt att olika metoders prestanda jämförs och att metoden testas med den typ av provmaterial som den senare ska användas för att analysera. Vi visar också att PCRs förmåga att upptäcka små mängder av T. gondii i ett prov fölbättrades när en del av parasitens arvsmassa som förekommer i 200-300 kopior per parasit analyserades istället för den del av arvsmassen som vanligtvis används och förekommer i 35 kopior. Här kunde även en ”intern amplifieringskontroll” användas, som direkt visar om PCRen vid något tillfälle inte fungerat som den ska. Provet måste då analyseras på nytt.

T. gondii delas vanligtvis in i tre huvudtyper som kallas typ I, II och III. Det är möjligt att dessa orsakar olika typer av sjukdomsförlopp vid toxoplasmos. Som
exempel har typ I kopplats samman med allvarliga ögoninfektioner i Sydamerika, medan typ II verkar vara den vanligast förekommande typen vid toxoplasmos hos människor i Europa. Rutinmässig identifiering av parasitens typ i patientprover kan öka våra kunskaper om vilken betydelse de olika typerna har vid toxoplasmos. I dagsläget saknas lämpliga metoder för detta. För detta ändamål har vi haft utvecklat en metod baserad på Pyrosequencing som kan utföras inom loppet av två till tre timmar, vilket kan jämföras med mer traditionellt använda metoder som tar betydligt längre tid att utföra.


Att med PCR övervaka patienter med nedsatt immunförsvar för att upptäcka toxoplasmos har flera syften: i) att upptäcka sjukdomen på ett tidigt stadium för att öka chanserna för att behandling ska lyckas ii) att analysera mängden av parasiter i ett prov för att undersöka hur detta hänger ihop med sjukdomsbilden, iii) att utvärdera olika behandlingars förmåga att motverka toxoplasmos, och iv) att vidare undersöka vilken roll parasitens typ spelar vid toxoplasmos.
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Molecular diagnosis of infection with Toxoplasma gondii in the immunocompromised patient


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