Diagnosis of infection with *Toxoplasma gondii* in pregnant women, neonates and immunocompromised patients

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Stockholm 2005

Published and printed by Repro Print AB

ISBN 91-7140-456-2

Abstract

Infection with *Toxoplasma gondii* poses unique diagnostic problems like long-term persistence of specific IgM-antibodies, which makes it difficult to use the presence of *Toxoplasma*-specific IgM-antibodies alone as a sign of acute infection. The importance of determining the time of infection in pregnant women is also a unique diagnostic challenge in *Toxoplasma* diagnostics. The first paper in this thesis compares the performance of different enzyme immuno assays, immunofluorescence assays, immunosorbent agglutination assays and IgG-avidity assays. The study showed that a combination of an IgM assay followed by an IgG-avidity test was the best combination to estimate the time interval in which infection had taken place. Diagnosis of infection in newborns without *Toxoplasma*-specific IgM- or IgA antibodies is difficult and a two-dimensional immunoblot assay to distinguish between maternal and child IgG-antibodies with different specificities was developed having higher sensitivity than previous assays. A new IgG-avidity assay based on recombinant antigens was developed, which effectively abolished the problem of long-term low IgG-avidity seen in samples analysed by assays using whole cell, lysed antigen. Enzyme immuno assays with whole cell, lysed antigen pose problems with poor discrimination between IgG negative and low-positive samples and recombinant antigens should provide assays with less background, however, the sensitivity may be reduced. Two studies show how combinations of recombinant antigens perform in assays of *Toxoplasma*-specific IgG- and IgM-antibodies. The assays do not yet have the same sensitivity as whole cell, lysed antigen based assays, but the concept is promising and should be further explored. *T. gondii* infection is a problem in immunodeficient hosts as the parasite remains alive in the chronically infected. Current strategies for diagnosing these infections rely on regular testing for *Toxoplasma*-specific DNA by PCR in blood and other fluids including bronchioalveolar lavage (BAL). We show that a new, real time PCR based assay can be used to detect *Toxoplasma* in BAL fluids from HIV patients.

This thesis is based on the following papers:

I. A. Roberts, K. Hedman, V. Luyasu, J. Zufferey, M.-H. Bessières, R.-M. Blatz, E. Candolfi, A. Decoster, G. Enders, U. Gross, E. Guy, M. Hayde, D. Ho-Yen, J. Johnson, B. Lécolier, A. Naessens, H. Pelloux, P. Thulliez, E. Petersen E. Multicentre evaluation of strategies for serodiagnosis of primary infection with *Toxoplamsa gondii*. Eur J Clin Microbiol Infect Dis 2001;20:467-74.

II. Beghetto E, Buffolano W, Spadoni A, Pezza M del, Cristina M di, Minenkova O, Petersen E, Felici F, Gargano N. Use of an Immunoglobulin G avidity assay based on recombinant antigens for diagnosis of primary *Toxoplasma gondii* infection during pregnancy. J Clin Microbiol 2003;41:5414-8.

III. Pietkiewicz H, Hiszczyńska-Sawicka E, Kur J, Petersen E, Nielsen H, Stankiewicz M, Andrzejewska I, Myjak P. Usefulness of *T.gondii* recombinant antigens in serodiagnosis of human toxoplasmosis. J Clin Microbiol 2004;42:1779-81.

IV. Henrik Vedel Nielsen, Dorte Remmer Schmidt, Eskild Petersen. Diagnosis of congenital toxoplasmosis by two-dimensional immunoblot differentiation of mother and child IgG-profiles. J Clin Microbiol 2005;43:711-5.

V. Petersen E, Thalib L, Gras L, Paul M, Wallon M, Evengard B, Hayde M, Gilbert R for the European Multicentre Study on Congenital Toxoplasmosis (EMSCOT). Screening for congenital toxoplasmosis: accuracy of IgM and IgA tests after birth. Submitted.

VI. Petersen E, Borobio MV, Guy E, Liesenfeld O, Meroni V, Naessens A, Spranzi E, Thulliez P. European multicentre study of the LIAISON® automated diagnostic system for determination of specific IgG, IgM and IgG-avidity index in toxoplasmosis. J Clin Microbiol 2005;43:1570-4.

VII. Petersen E, Edvinsson B, Benfield T, Lundgren B, Evengård B. Diagnosis of pulmonary infection with *Toxoplasma gondii* in immunocompromised patients. Mansucript.

VIII. Buffolano W, Beghetto E, Del Pezzo M, Spadoni A, Di Cristina M, Petersen E, Gargano N. The use of recombinant antigens for the early postnatal diagnosis of congenital toxoplasmosis. Submitted.

Contents

Abbreviations

2DIB	Two-Dimensional Immunoblot
BAL	Bronchioalveolar Lavage
BMT	Bone Marrow Transplant
CT	Congenital Toxoplasmosis
CFA	Complement Fixation Assay
CSF	Cerebrospinal fluid
EIA	Enzyme Immuno Assay
EMSCOT	European Multicentre Study on Congenital Toxoplasmosis
DT	Sabin Feldman's dye test
GRA	Dense Granule Antigens of *Toxoplasma gondii*
HAART	Highly Active Anti-Retroviral Therapy
IFA	Immunofluorescence Assay
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
MIC	Microneme Antigens of *Toxoplasma gondii*
ISAGA	Immunosorbent Agglutination Assay
PCR	Polymerase Chain Reaction
RecEIA	Recombinant antigen EnzymeImmuno Assay
SAG	Surface Antigens of *Toxoplasma gondii*

Introduction

The diagnostic challenges

Infection in pregnant women with *Toxoplasma gondii* may be transmitted to the fetus where it may cause permanent damage of the fetus including retinochorioditis and hydrocephalus. The infection may reactivate after birth with new attacks of retinochorioditis and reduced eye sight as the result.

Infection of the woman before pregnancy causes immunity and the infection is transmitted to the fetus, and therefore it is essential to estimate the time of infection as precisely as possible to properly estimate the risk of infection for the fetus.

Congenital infection of the fetus in women infected just before conception is extremely rare and even during the first few weeks of pregnancy the maternal-fetal transmission rate is only a few percent (Dunn et al. 1999).

Strategies for control and prevention of congenital toxoplasmosis vary between countries and the diagnostic challenges are different in pre- and neonatal screening programmes.

Systematic, prenatal screening is performed in Austria, France and Slovenia and widespread on-demand screening takes place in Belgium, Germany, Italy and Spain. Samples are obtained during pregnancy and analysed for *Toxoplasma*-specific IgM- and IgG-antibodies. When seroconversion is detected, the mother is infected and treatment is usually started.

The biggest diagnostic challenge is the situation when *Toxoplasma*-specific IgG and IgM-antibodies are found in the first sample after conception, where the time of infection is the key to estimate whether the fetus is at risk or not (Ades 1991).

Neonatal screening for congenital toxoplasmosis is performed in New England, Denmark and parts of Brazil by analyzing the blood samples obtained on filter paper (Guthrie cards) day 5 postpartum (Guerina el al. 1994; Lebech et al. 1999; Neto et al. 2004). Detection of *Toxoplasma*-specific IgM-antibodies eluted from the PKU-filter paper is followed by a request of a blood sample from both the mother and infant for confirmatory testing (Sorensen et al., 2002). Fifteen to twenty percent of these sera are found to be negative for *Toxoplasma*-specific-IgM (Decoster et al. 1992; Lebech et al. 1999; Naot et al. 1981).

Low levels of *Toxoplasma*-specific IgM antibodies may be found for up to several years after acute infection, and the mere demonstration of low levels of *Toxoplasma*-specific IgM-antibodies is therefore not regarded as a sign by itself of acute infection with *T. gondii* (Liesenfeld et al. 1997, Liesenfeld et al. 2001a,b).

The measurement of the avidity of IgG-antibodies was first demonstrated for *T. gondii* infections in 1989 (Hedman et al. 1989, Hedman et al. 1993, Lecolier & Pucheu 1993) and has since then been further developed (Dannemann et al. 1990, Marcolino et al. 2000; **II**; **VI**) but does not work in the beginning of the infections with low levels of IgG-antibodies (Press et al., 2005).

A study of the diagnostic value of different diagnostic tests for acute infection with *T. gondii* including *Toxoplasma*-specific IgG-, IgM- and IgA-antibodies and the IgG avidity index showed that the combination of a sensitive test for *Toxoplasma*-specific IgM-antibodies and a *Toxoplasma*-specific IgG avidity assay had the highest predictive value of the time of infection (**I**).

Since previous studies have shown that some individuals have low avidity-IgG antibodies many months after infection (**VI**), we also tested the hypothesis whether treatment influences the maturation of IgG-antibodies and based on just twelve untreated patients, it seems that treatment and or pregnancy may delay the IgG-maturation (**VI**).

Biology, history and transmission

T. gondii is a protozoan parasite found worldwide and able to infect all mammals and birds. The parasite was first described in by Nicolle and Manceaux and Splendore (1908). *T. gondii* has three main life stages: the oocysts excreted in feces from felines, the actively dividing tachyzoites found during an acute infection or activation and the bradyzoites or tissue cysts, which are found in muscles, brain and other tissues in infected animals for the rest of their lives.

Felines are the main hosts where the sexual part of the life cycle takes place in the intestine, and felines can excrete millions of oocysts in their feces for weeks after the acute infection. Oocysts may remain alive and contagious for several months in the environment, the length depending on temperature and humidity (reviewed by Petersen & Dubey 2001).

Intermediate hosts like other mammals including man and birds are infected either by eating raw *T. gondii* infected meat or by ingesting oocysts, and in Europe approximately two-thirds of *T. gondii* infections in pregnant women are estimated to come from raw and undercooked meat (Cook et al. 2000).

Molecular biology

T. gondii is a complex protozoan parasite with three distinct life-stages, each with stage-specific expression of antigens (Kasper and Ware, 1989; Singh et al. 2002). The most immunodominant antigen is the tachyzoite-specific Surface Antigen 1, SAG1, (previously known as P30) which comprise up to 5% of the protein of the tachyzoite (Burg et al. 1988). The antigen expressed in *E. coli* has been shown to be recognised by natural SAG1-antibodies (Harning et al. 1996), and SAG1 is considered a prime candidate antigen in diagnostic tests because of its immunodominance and lack of known cross-reactivity to antigens from other microorganisms.

Other surface antigens, SAG2, SAG3 and SAG4 have been identified. SAG2 and SAG3 being tachyzoite specific and SAG4 bradyzoite specific (Cesbron-Delauw 1995; Howe and Sibley 1994; Cesbron-Delauw et al. 1994; Odberg-Ferragut et al. 1996). Two other groups of *T. gondii* antigens have been studied for use in diagnostic assays, the dense granule antigens, GRA's, and in particular GRA1 and GRA6 (Lecordier et al. 2000) and GRA7 (Fischer et al. 1998) and microneme antigens, MIC's (Cerede et al. 2002; Garcia-Règuet et al. 2000; Lourenco et al. 2001). The MIC antigens have also been shown to be important in the induction of protective immunity (Beghetto et al. 2005).

Bradyzoite-specific antigens like the bradyzoite antigen 1, BAG1 (Bohne et al. 1993), and matrix antigen 1, MAG1 (Parmley et al. 1994) should in theory be important in the antibody repertoire in infections past the acute stage, but they still remain to find their place in future diagnostic assays.

The diagnostic value of oocyst specific antigens has been studied in a single study of *T. gondii* oocysts infected cats, but has not yet been tested in humans (Dubey et al. 1995).

Importance in humans

Immunocompetent patients

The first human case ascribed to infection with *T. gondii* was a child with hydrocephalus reported by Janku in 1923 (Janku 1923).

Sabin reported the first case of encephalitis due to *T. gondii* (Sabin 1941), and encephalitis due to *T. gondii* in immunocompromised patients was first reported from patients with Hodgkin's disease during immunosuppressive treatment (Flament-Durand et al. 1967).

During the nineteen forties there was improved understanding of the cause of maternal infection for congenital toxoplasmosis in newborns, and in 1953, Feldman reported a series of 103 children of which 99% had eye lesions, 63% had intracranial calcifications and 56% had psychomotor retardation (Feldman 1953).

This initiated interest in congenital infection among scientists in Europe (Couvreur 1955). In Gothenburg, Sweden, 50% of mothers had had previous infection with *T. gondii* and two out of 23,260 children had clinical toxoplasmosis during a 1948 – 51 study period (Holmdahl & Holmdahl 1955). A study from Austria reported frequent symptoms in children with congenital toxoplasmosis (Eichenwald 1957). A French study concluded that treatment prevented transmission from mother to child and reduced the clinical symptoms in children (Couvreur and Desmonts 1962), and another study from France showed that the seroprevalence in pregnant women in Paris was 85% with a high risk of *Toxoplasma* infection in sero-negatives (Desmonts et al. 1965). This was followed by a larger study from France of 374 pregnancies (Desmonts and Couvreur 1974).

In France and Austria prenatal screening programmes were introduced in 1975 (Thulliez 1992; Aspöck and Pollak 1992). In Austria three samples are analysed during pregnancy (Thalhammer 1975; Aspöck and Pollak 1992), and in France monthly screening is performed in seronegative pregnant women (Thulliez 1992). Presently, prenatal screening is performed widely in Belgium, Germany, Italy and Spain although not through organized systematic programmes.

The use of *Toxoplasma*-specific IgM-antibodies for neonatal diagnosis was propose in 1968 (Remington et al., 1968), and systematic neonatal screening was piloted in New York (Kimball et al. 1971). The first neonatal screening programme based on detection of IgM antibodies at birth was initiated in the New England states in 1988 (Guerina et al. 1994).

Neonatal screening was again evaluated in Denmark in 1992 – 96 and approximately 75% of *Toxoplasma*-infected newborns were found to have detectable *Toxoplasma*-specific IgM-antibodies at birth (Lebech et al. 1999). Based on these results the Danish National Board of Health recommended a neonatal screening programme for congenital toxoplasmosis, which started the 1st of January 1999.

Immunocompromised patients

HIV-infected patients

The increased frequency of *Toxoplasma*-encephalitis in patients with AIDS was reported soon after the start of the HIV-epidemic (Roue et al. 1984; Enzensberger et al. 1985; Suzuki

et al. 1988) and *Toxoplasma*-encephalitis was an important end-stage cause of death in HIV-patients before the introduction of HAART (Highly Active Anti-Retroviral Therapy).

Cardiac and kidney transplants

The majority of *T. gondii* infections in immunocompromised hosts are reactivations of previous infections (Mele et al. 2002). The seroprevalence is low in northern Europe, less than 2% in northern Sweden (Forsgren et al., 1991, Evengard et al., 2001), and higher further south up to 60% in Poland (Paul et al., 2000), and is strongly age-dependent.
In patients receiving a cardiac transplant, six weeks pyrimethamine prophylaxis reduced infection from 57% (4/7) to 14% (5/37) (Wreghitt et al., 1992). A review of 257 heart transplants 1985 – 1993 and 33 heart-lung transplants found that 4.5% (13) were donor *Toxoplasma* positive, recipient negative of which 9 were followed up and only one patient seroconverted. All patients received trimethoprim/sulfamethoxazole prophylaxis for *Pneumocystis* (Orr et al., 1994). A later study clearly showed the risk of infection in *T. gondii* naïve recipients receiving a cardiac transplant from a *T. gondii* positive donor of which 78% seroconverted (14/16), in contrast only 10% (6/59) developed serological evidence of *Toxoplasma*-infection in donor negative-recipient positive cases (Gallino et al. 1996). *Toxoplasma*-infection has also been described after kidney transplants (Aubert et al., 1996; Giordano et al., 2002; Renoult et al., 1997, Wulf et al., 2005).

Bone marrow transplants

The prevalence of *T. gondii* in BMT patients also varies with the seroprevalence in the population (0.5% in the U.S. to 5% in France). A review of 55 patients with allogenic BMT with *T. gondii* infection found that only 4% survived (Chandrasekar et al. 1997). Symptoms of *T. gondii* infections in bone marrow transplant patients include fever (43%), seizures (14%), headache (13%), confusion (13%) and pulmonary symptoms (12%). 92% had more than one symptoms and the average onset 62 days post BMT (range 1 – 689) (Mele et al. 2002).
The European Group for Blood and Bone Marrow Transplantation reported on 106 allogenic stem cell transplants of which 55% of the donors were *Toxoplasma* IgG positive. All received prophylaxis with trimethoprim and sulfamethoxazole for six months and 15% (16/106; 95%CI: 8% - 21%) had at least one *T. gondii* PCR positive blood sample and 6% (6/106; 95% CI: 1% - 10%) experienced clinical disease due to *T. gondii* (Martino et al. 2005). The median days to diagnosis from onset of symptoms was 42 days (range 1-178 days) and the presenting symptoms were localized encephalitis in 4 patients, pulmonary toxoplasmosis in one and one presented with acute disseminated disease (Martino et al. 2005).

The historic development of diagnostic assays

The complement fixation assay, CFA, was the first diagnostic test for *Toxoplasma*-specific antibodies (Warren and Sabin 1941; Steen and Kaas 1951). The dye-test described by Sabin and Feldman (1948) is based on antibody-mediated killing of live *T. gondii* parasites in the presence of complement. If antibodies were present in the sample, the parasites are made penetrable for methylene blue and are coloured in the presence of complement, if antibodies are not present, the parasites remain unstained against the blue background of the methylene dye. The dye-test, DT, has proved a very sensitive assay, but the requirement of live, *T. gondii* parasites makes it difficult and expensive to perform, and the test is now only performed in a few reference laboratories (Reiter-Owona et al. 1999). The DT is not included in reference panels circulated as part of external quality control programmes, and multicentre studies show a considerable variability (Pethithory el al. 1996; Reiter-Owona et al. 1999; Rigsby et al. 2004). Immunofluorescence assays, IFA, were introduced in the 1960'ies (Ambroise-Thomas et al. 1966) and proved specific, but with a lower sensitivity compared to the DT. The IFA for *Toxoplasma*-specific IgM-antibodies is still used by some centres because it is highly specific, but it has a low sensitivity (**I**).

The Enzyme Immuno Assay technique, EIA, became available in 1972 (Engvall & Perlmann, 1972). The first *Toxoplasma*-specific IgM-assay was developed by Remington and Miller (1966) and the first EIA based assay by Naot and Remington (1980). By the end of the 1980'ies the direct EIA measuring *Toxoplasma*-specific IgG-antibodies and the μ-capture EIA measuring *Toxoplasma*-specific IgM-antibodies were well established in reference centres and the first commercial test produced by industry had been introduced (Schaefer et al.1989). The μ-capture IgM assays were an improvement over the direct EIA IgM assays, but still had problems with false positive results. The development of the Immunosorbent Agglutination assays, ISAGA, solved this by using whole cell formalin fixed *T. gondii* and tests based on this technique are regarded as highly sensitive and specific for *Toxoplasma*-specific IgM- and IgA-antibodies (LeFichoux et al. 1984; Pouletty et al. 1984). Western blot to single antigens was tested to improve diagnostic sensitivity (Gross et al. 1992).

A method to measure the maturation of *Toxoplasma*-specific IgG-antibodies to determine the time of infection was described by Hedman et al. (1989). The test explores the increasing avidity by the specific IgG-antibodies with the maturation of the immune response, and in the original study it was shown that the time of infection could be determined within a three months window after infection. The test has later been adapted to

automated systems (**VI**). The same principle is used in the differentiated agglutination test (Thulliez et al. 1989).

Aims of the study

Main aim

- to improve diagnosis of *T. gondii* infection in pregnant women, newborn children and immunosuppressed individuals.

Specific aims

- to provide knowledge of the natural history of the *Toxoplasma*-specific antibody response, with special emphasis on the IgM-response and the development of IgG-avidity;
- to evaluate whether treatment influences IgG-avidity maturation;
- to evaluate the performance of diagnostic assays in relation to the time of infection in pregnant women;
- to evaluate the performance of diagnostic assays in newborns with congenital toxoplasmosis;
- to evaluate new diagnostic tools based on recombinant *Toxoplasma*-antigens in pregnant women and newborns with congenital *Toxoplasma*-infection;
- to evaluate new diagnostic tools based on detection of *Toxoplasma* nucleic acid in immunocompromised patients.

Patient and Methods

Patients

Patients from several cohorts were included in the different studies. Clinical data were available on the patients from whom the samples were obtained, which made it possible to relate the test results to the clinical situation.

A European multicentre study provided samples from seroconverting women only, which were used in study **I**. In the prospective cohort study **II** and **VIII** sera were included from women with *Toxoplasma*-infection during pregnancy identified by prenatal screening in Campagnia region, Italy. Study **IV** used samples from mothers and newborns identified by the Danish neonatal screening programme, and additional samples from seroconverting pregnant women from Gdansk, Poland in study **III**.

Study **V** was based on the EMSCOT cohort (European Multicentre Study on Congenital Toxoplasmosis), an EU funded, prospective study including 13 European centres, and study **VI** was a separate cohort of samples from pregnant and non-pregnant patients with acute *Toxoplasma* infection from six European centres. Study **VII** included BAL samples from HIV-infected patients from Copenhagen with advanced HIV-infection.

Methods

Antibody measurements

The methods used for measuring a *Toxoplasma*-specific IgG- or IgM immune response included both commercial and in-house tests of *Toxoplasma*-specific IgM, IgA and IgG-antibodies and *Toxoplasma*-specific IgG-avidity index. Reactivity was measured by enzyme immunoassays, EIA, immunosorbent agglutination assay, ISAGA, Sabin Feldman's dye test, immunofluorescence, IFA, agglutination assays and IgG-avidity assays based on elution of low-avidity, *Toxoplasma*-specific IgG-antibodies by urea.

The individual papers describe the development of diagnostic assays during the study period from using crude, whole cell, lysed *T. gondii* antigens towards single and combinations of different recombinant *T. gondii* antigens for both EIA and IgG-avidity tests.

Nucleic acid detection

Real time PCR was used to detect *Toxoplasma*-specific nucleic acid in bronchioalveolar lavage from immunosuppressed, HIV-infected patients from the Copenhagen HIV cohort.

Results and Discussion

Diagnosis of *Toxoplasma gondii* infection in pregnant women

In countries where pre-natal screening programmes are in place a test of the first blood sample from the pregnant women for *Toxoplasma*-specific IgM- and IgG-antibodies is performed. Approximately 5% of seropositive women in the first trimester have *Toxoplasma*-specific IgM-antibodies (**V**), but only approximately 4% of these give birth to a child with congenital *Toxoplasm*a infection. It is therefore a considerable problem to diagnose whether women with specific IgM antibodies are infected before or after conception. This is particularly a problem in countries where testing of pregnant women in the beginning of pregnancy are common.

This problem has been partly solved by obtaining two samples from pregnant women to see if there is any development of the immune response. It is generally agreed that there is a development of the *Toxoplasma*-specific IgG-antibody response within the first 8 weeks after infection after which the IgG levels are maintained at a high level, with or without declining IgM antibodies (Jenum et al. 1997; Jenum et al. 1998).

The question of too many low-level *Toxoplasma*-specific IgM-positive patients and whether the diagnostic performance could be improved by not merely repeating the same tests two weeks apart, led us to design a study where the sensitivity and specificity of the different assays and their ability to predict recent infection were assessed (**I**).

IgG-avidity index

In a European multicentre study many laboratories contributed samples from patients in whom the time of infection was known (**I**). This panel was used to determine the proportion of sera showing specific IgM and IgA antibodies to *T. gondii* as well as the IgG-avidity index within one to three months, three to twelve months or more than twelve months after seroconversion. These data were used to propose a two level strategy for diagnosis. (**I**)

Combined, two-test strategies

The study (**I**) showed that the best strategy for diagnosing acute and recent infection with *T. gondii* was a two-test strategy with a sensitive IgM test first followed by an IgG-avidity test (**I**). Thus the study confirmed the need of the *Toxoplasma*-specific IgG-avidity index assay in the diagnosis of acute and recent infection.

The increased use of the *T. gondii* IgG-avidity test has highlighted an inherent problem with the test that many pregnant women have long-lasting low IgG-avidity antibodies (**VI**) and the IgG-avidity assay needs further development which could be by the use of recombinant antigens (**II**).

Improvement of EIA tests for *Toxoplasma*-specific IgG- and IgM-antibodies

The problems with IgM-based diagnostics in *T. gondii* infections have resulted in attempts to improve the tests. The accepted reference test is the ISAGA, but most analyses are performed with an EIA μ-capture test. The assays use whole-cell, lysed *T. gondii* as antigen, and attempts have been made to improve the test by using recombinant antigens (Ferrandiz et al., 2004). The ISAGA IgM and EIA IgM and IgM immunofluorescence were evaluated in a prospective, European cohort study, EMSCOT, of women diagnosed with primary *T. gondii* infection during pregnancy and newborns identified through neonatal screening (**V**).

The EMSCOT study provided data on sensitivities for diagnosing congenital infection in the newborn of four *Toxoplasma*-specific IgM-antibody assays and three *Toxoplasma*-specific IgA-antibody assays. The study also provided data on the sensitivity of neonatal testing related to the estimated gestational age of infection with *T. gondii*, showing that the IgM-seropositivity at birth only detects infections in the second half of pregnancy (**V**).

The study included 5,223 samples from 996 children of which 3,742 were tested with an EIA system, 2,011 with an ISAGA IgM and 316 with the IgM immunofluorescence assay (**V**).

The children were followed for one year to ascertain the diagnosis by demonstrating the presence of *Toxoplasma*-specific IgG-antibodies at 12 months of age, which is the golden standard for confirming congenital infection with *T. gondii*.

With this as the reference the sensitivity and specificity of the different blood tests taken at birth is shown in table 1 below. The sensitivity for EIA tests is low, 29.3%, clearly demonstrating the need for better tests.

Table 1. Sensitivity and specificity of IgM tests at birth (**V**)

	Sensitivity (95% CI)	Specificity (95% CI)
ISAGA IgM	0.536 (0.45, 0.62)	0.964 (0.95, 098)
ELISA IgM	0.293 (0.17, 0.46)	0.963 (0.93, 0.98)
IFAT IgM	0.100 (0.01, 0.46)	1.000 (0.92, 1.0)

Table 2 shows the same data according to the gestational age of infection.

Table 2. Sensitivity and specificity of IgM tests according to gestational age at infection (**V**).

IgM	Sensi-tivity	Speci-ficity	Post test probability for CT given a pos. test	Post test probability for CT given a neg. test
Trimester 1 0-14 weeks +6 d	0.36	0.96	0.23	0.02
Trimester 2 15w to 27w+6d	0.33	0.97	0.81	0.20
Trimester 3 28+IgM	0.71	0.89	0.90	0.32

CT= congenital toxoplasmosis

The study was the first study to provide data on the sensitivity of *Toxoplasma*-specific IgM-antibodies related to gestational age at infection. It is clearly seen that a sensitivity of 50% is not reached until after the 30th gestational week.

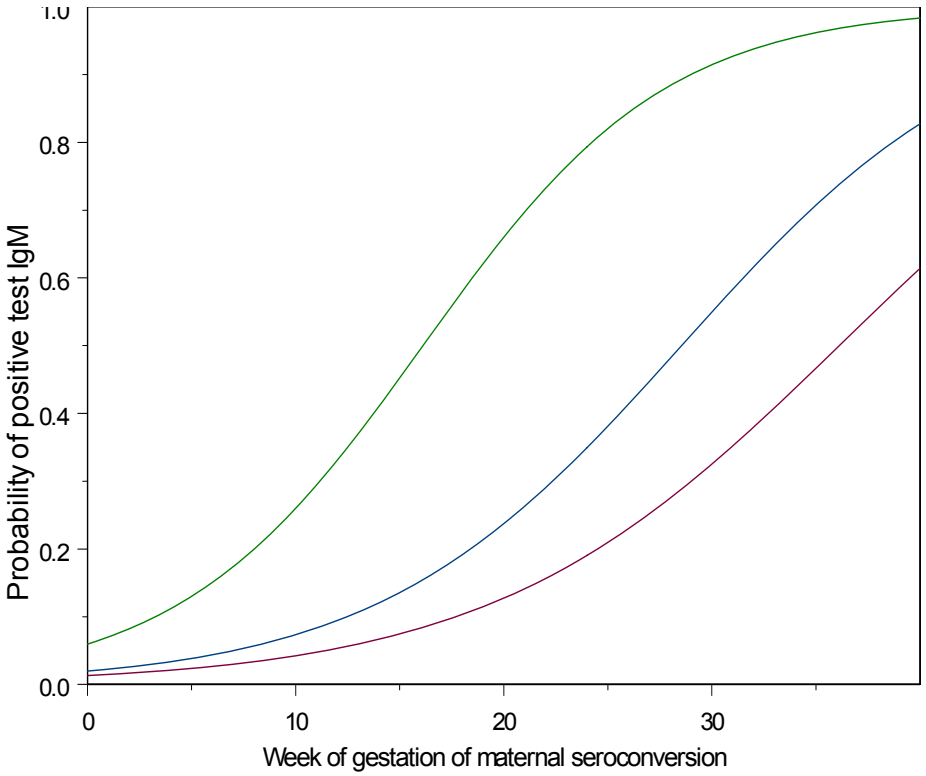

Figure 1.

The post test probability of a positive IgM-test at birth related to the gestational age at infection with the 95% confidence interval (**V**).

Recombinant IgG-assays, adults

Conventional assays have so far used whole-cell, lysed, *T. gondii* antigens, which have batch variations. With increasing emphasis on need for reproducibility the use of recombinant antigens in diagnostics assays provide a theoretical advantage.

Previous studies have shown that the GRA1, GRA7 and SAG1 molecules are immunodominant (Aubert et al. 2000; Harning et al. 1996; Jacobs et al. 1999; Johnson et al. 1992; Li et al. 2000). Our study showed that recombinant antigens including a mixture of GRA1, GRA7 and SAG1 were not as sensitive as whole-cell, lysed, antigen if sera had an IgG titer of less than 1:1,600 using an EIA test and less than 1:512 in an IgG-immunofluorescence test (**III**). The test did, however has 100% sensitivity in a panel of sera from individuals who had *Toxoplasma*-specific-IgM- and/or IgA-antibodies indicating that the infection was recent (**III**). Future assays for *Toxoplasma*-specific IgG-antibodies relying on recombinant antigens need to include a panel of antigens and the test with recombinant antigens has not yet been optimized to the same sensitivity as the whole-cell, lysed antigen assay.

Recombinant IgM- and IgG-assays, newborns

Diagnostic assays based on recombinant antigens for measuring the *Toxoplasma*-specific IgM-antibodies were evaluated in infants with or without congenital toxoplasmosis born to mothers with toxoplasmosis acquired during pregnancy (**VIII**).

Antigen fragments from the MIC2, MIC3, M2AP, and SAG1 protein were tested in an EIA test (RecEIA) on 104 serum samples from newborns born to mothers infected with *T. gondii* during pregnancy. Thirty-five were congenitally infected and thirty-four out of 35 (97%) serum samples from the congenitally infected patients reacted with at least one of the recombinant antigens (**VIII**).

Remarkably, all sera from 22 *Toxoplasma*-infected newborns which were clinically and serologically undiagnosed at birth were reactive using the IgM Rec-ELISA analysis, allowing to confirm congenital toxoplasmosis as soon as two months after birth. The presence of *T. gondii*-specific, IgM antibodies against recombinant MIC2, MIC3, M2AP, and SAG1 antigens may be used for the early postnatal diagnosis of congenital toxoplasmosis.

It was also found that the newborn, *Toxoplasma*-infected child primarily produces IgG_2 and IgG_3 against recombinant *Toxoplasma*-antigens, whereas the maternally transferred antibodies primarily were IgG_1 (**VIII**). Thus subclass analysis of serum samples from mother and child against defined recombinant antigens may further improve diagnosis of congenital *Toxoplasma*-infection in newborns.

The *Toxoplasma*-specific IgG-avidity index

The maturation of the IgG-response varies considerably between individuals. In the study of Lappalainen et al. (1993) two seroconverting mothers already had an IgG-avidity index above 20% at the time of diagnosis, but most patients had developed an IgG-avidity index above 15% after 180 days (Lappalainen et al. 1993). A study from France found an average IgG-avidity index of 0.2 in pregnant women infected within 5 months (Leolier & Pucheu 1993).

The original method developed by Hedman et al. (1989) used serial dilutions tested in EIA with and without 6M urea, but automated assays today calculate the IgG-avidity index from two single measurements of the sample with and without urea. This introduces an uncertainty, although experiments with only two serum sample dilutions showed an excellent agreement with IgG-avidity measurements using 4 serial serum sample dilutions (Korhonen et al. 1999). Prince and Wilson (2001) evaluated the IgG-avidity assay and found that using single dilution assays with and without urea and showed that because the signal obtained in an EIA system is not linear, it makes a difference whether the *Toxoplasma* IgG-avidity index is calculated from the OD values or the activity measured in International Units of *Toxoplasma*-specific IgG-antibodies per ml (Prince & Wilson 2001).

The IgG-avidity results found in one of our studies (**VI**) showed that persistent, low IgG-avidty index poses a diagnostic problem, at least in pregnant women receiving treatment during pregnancy (**VI**).

Up to half of the patients with acute infections may show a low or borderline IgG-avidity index six months after the infection (Montoya et al. 2002; Rossi 1998) which is in concordance with the results reported in our study (**VI**). The LIAISON® avidity results were compared with the semi-automated VIDAS system for measuring the *Toxoplasma*-specific IgG-avidity index and there was a good correlation between the results from the two systems showing that persistence of low-level *Toxoplasma*-specific IgG-avidity antibodies is an inherent problem of measuring the *Toxoplasma*-specific IgG-avidity index unrelated to the assay system.

The cut-off value defining a low IgG-avidity index differs markedly between different studies and one study found that patients infected within the past three months had IgG-avidity

index below 0.45 (Holliman et al. 1994). A comparison between the VIDAS and the Labsystems IgG-avidity index showed a correlation coefficient of 0.6 in pregnant women but 0.88 in other patients (Alvarado-Esquivel et al. 2002), but the difference was not further discussed. Improvement of the IgG-avidity assay using Western blot technique has been attempted and revealed differences in the maturation of the specific IgG to different antigens (Villavedra et al. 1999).

The IgG response matures rapidly in some individuals and this has been reported in several studies. For instance, the cut-off of the *Toxoplasma*-specific IgG-avidity index using the VIDAS system (bioMérieux) was defined as 0.3 to ensure that all sera from acute infections had a low-avidity index (Pelloux et al., 1998). The same study showed that at least in pregnant women, a low IgG-avidity index persisted up to 9 months post-infection and all women were treated. In a study of *T. gondii*-infected pregnant women identified prospectively through prenatal screening, Jenum et al. (1997) found that 2 out of 73 women had an IgG-avidity index above 0.2 before 20 weeks of gestation, but many continued to have a low IgG-avidity index even a year after infection. It is assumed that all women were treated during pregnancy.

The IgG-avidity results found in one of our studies (**VI**) showed that long lasting low IgG-avidity was common in pregnant women. The study compared the IgG-avidity maturation in treated, pregnant women with samples from patients with acute infection with *T. gondii* who were not pregnant and were not treated, and found a significantly more rapid IgG maturation during the first four months after infection in subjects who were not treated and not pregnant (**VI**).

The observation that the *Toxoplasma gondii*-specific IgG-maturation is delayed in treated pregnant women compared to non-treated, non-pregnant individuals has been reported in one previous study, which found a significantly delayed IgG-maturation in treated individuals (Sensini et al. 1996). Our finding that treatment may influence the IgG-avidity maturation underlines the need for further studies to better clarify the avidity maturation process in pregnant women under therapy in comparison with the untreated individuals. If confirmed different cut-off values will have to be defined for treated and untreated and/or pregnant and non-pregnant individuals.

Diagnosis of *Toxoplasma gondii* infection in live-born neonates

Diagnosis of congenital infection with *T. gondii* is difficult at birth if *Toxoplasma*-specific IgM- and/or IgA-antibodies are not present, because present diagnostic methods can only with difficulty distinguish between maternal and fetal IgG. The traditional method of diagnosing congenital toxoplasmosis in IgM and IgA negative newborns is to wait up to 12 months and

observe if the maternal *Toxoplasma*-specific IgG antibodies disappear. If the child has been treated continuously with sulfadiazine and pyrimethamine the synthesis of *Toxoplasma*-specific IgG-antibodies can be suppressed and the serological confirmation of the infection can sometimes not be made with certainty before the second year of life (Wallon et al. 2001). This situation is found when *T. gondii* infection is suspected but *Toxoplasma*-specific IgM and/or IgA antibodies can not be demonstrated in the child and parasitological investigations like PCR analysis for *Toxoplasma*-specific nucleic acid is negative or not appropriate. IgM- and IgA antibodies do not cross the placenta, and neonatal screening programmes for congenital toxoplasmosis are based on detection of *Toxoplasma*-specific IgM-antibodies eluted from blood spots from PKU-filter papers (Guthrie-cards) (Guerina et al. 1994; Lebech et al. 1999; Neto et al. 2004; Sorensen et al., 2002). Different cut offs for maternal and newborn *Toxoplasma*-specific IgM antibodies has been proposed (Candolfi et al., 1993). It has been hypothesized that treatment of acute toxoplasmosis during pregnancy reduced the duration of the *Toxoplasma*-specific IgM response, but two studies did not find such an effect (Gras et al., 2004; **V**).

Demonstration of *Toxoplasma*-specific IgG-antibodies with different specificities in sera from the mother and child shows that the child synthesizes its own IgG-antibodies, confirming that the child is infected with *T. gondii.*

Previous studies have shown that transferred maternal and neo-synthesized *T. gondii*-specific IgG-antibodies can be differentiated by immunoblot or immunocomplexing (Chumpitazi et al. 1995; Gross et al. 2000; Remington et al. 2004; Robert-Gagneaux et al. 1999). Differentiation of the specificities of IgG-antibodies in the mother and child can also be done by comparing *T. gondii* antigen precipitated with maternal or child sera before performing an electrophoresis of the antigen-antibody complex (Pinon et al. 1996; Pinon et al. 2001; Robert-Gangneux et al. 1999).

The Western blot technique and immunocomplexing were compared in a double blind study and found to be equally sensitive (Pinon et al. 2001). The Immunoblot technique identifies newborns with congenital toxoplasmosis with a sensitivity of approximately 70% (Rilling et al. 2003; Tissot Dupont et al. 2003) increasing to 85% within the first 3 months of life (Gross et al. 2000; Rilling et al. 2003; Tissot Dupont et al. 2003). These results still leave 15-30 % of congenitally infected newborns without a confirmed diagnosis.

To improve the diagnosis of congenital toxoplasmosis a two-dimensional immunoblot, 2DIB, assay was developed capable of distinguishing between maternal and neonate *Toxoplasma*-specific IgG with a better sensitivity than previous assays (IV). The two-dimensional immunoblot, 2DIB, methodology greatly increased the resolution of the antibody

response by allowing identification of up to a thousand spots where the most sensitive Western blots do not allow distinction of less than fifty bands, often considerably less (**IV**).

Immunocompromised patients

Pulmonary toxoplasmosis is well known in HIV infected patients with low CD4+ T cell counts (Rabaud et al., 1996). The study of bronchioalveolar lavage, BAL, samples from 332 Danish HIV-infected patients found 2.1% (7/332) positive samples using a new, sensitive real-time PCR method (**VII**). The patients were in an advanced stage of immunosuppression with a mean $CD4^+$ T cell count of 39 x 10^6 per liter (range 0 and 161 x 10^6 per liter; normal values > 650 x 10^6 per liter). Records were retrieved from 6 out of the 7 patients, and all had pulmonary symptoms including dyspnoea and cough. Four out of six patients had interstitial infiltrates on chest X-rays and these four also had *P. jirovecii* in the BAL fluid. Information on fever was available from five patients of which two were febrile at the time of sampling. Four of the six patients had cough and or dyspnoe and one received oxygen treatment (**VII**).
T. gondii infections in cardiac transplants can be managed by careful prevention with treatment of the recipient.
Monitoring bone marrow transplant patients by PCR on peripheral blood, BAL fluid and CSF (according to local symptoms), treatment with pyrimethamine has reduced mortality to the same levels as for *T. gondii* negative BMT patients. The same strategy can be applied to other immunosuppressed patients in risk of developing *T. gondii* infection including HIV patients with low $CD4^+$ T cell count.
Trimethoprim/sulfamethoxazole is used routinely for prevention of P*. jirovecii* in HIV-infected patients with CD4+ T cell counts below 200 x 106 per liter. An early study showed that trimethoprim/sulfamethoxazole was prevented *T. gondii* infection in most but not all immunosuppressed patients (Norrby et al. 1975), results which are supported by our data showing that *T. gondii* infection in individuals despite trimethoprim7sulfamethoxazole treatment. Thus *T. gondii* infection should be suspected in immunosuppressed patients with unspecific symptoms of infection irrespective of trimethoprim/sulfamethoxazole prophylaxis. The most important factor in the management of the immunosuppressed patient is to consider *T. gondii* as a potential causative agent in patients presenting with unspecific symptoms including focal symptoms from CNS, heart, lungs and liver. Serology has been replaced by PCR analysis of *T. gondii* specific nucleic acid.

Conclusions and perspectives

Time of infection in pregnant women

Conclusions

- With the present diagnostic assays of *T. gondii* infections it is not possible to determine a precise time of infection in pregnant women.
- The development of the two step diagnostic algorithm using a combination of a sensitive IgM method followed by an IgG-avidity assay was a step forward, and the introduction of the recombinant IgG-avidity assay shows that long-term low IgG-avidity reactions can be solved.
- Treatment and/or pregnancy may prolong the IgG-avidity maturation.

Perspectives

- Further development of IgG-avidity assays using recombinant antigens is needed and the time window of four months before high avidity IgG antibodies are found should be reduced.
- Assays based on recombinant antigens should be improved to reduce unspecific background, improve sensitivity and exploit the kinetics of the antibody response during infection.
- The influence of treatment and/or pregnancy on IgG-avidity maturation s further studies.

Diagnosis of congenital toxoplasmosis in newborns

Conclusions

- The existing *Toxoplasma*-specific IgM assays applied to sera from newborns showed a low sensitivity.
- Neonatal screening based on detection of *Toxoplasma*-specific IgM-antibodies at birth has a sensitivity of less than 50% in children infected before the 30th gestational week and hardly detect any child infected before gestational week 20.
- The use of differentiated immunoblots like the novel two-dimensional immunoblot can in some congenitally infected children provide a diagnosis within a few weeks after birth.

- Newborns with congenital toxoplasmosis had a different subclass distribution of *Toxoplasma*-reactive IgG-antibodies compared to maternal transferred IgG.

Perspectives

- The combination of recombinant antigens and a different IgG-subclass response by the mother and the newborn child should be explored for diagnosis of congenital toxoplasmosis.
- Screening programmes based on neonatal detection of *Toxoplasma*-specific IgM needs improved assays.
- Immunoblots comparing epitope recognition by mother and child sera need further development which could be by using panels of recombinant antigens.

Diagnosis of infection in immunocompromised patients

Conclusions

- Detection of *Toxoplasma*-specific DNA by real-time PCR in bronchioalveolar lavage in immunocompromised hosts is a sensitive tool to reveal active infection, but needs to be repeated at regular intervals during the period of immunosuppression.
- The assay should be included in the panel of tests performed in the febrile, multisymptomatic immunocompromised host.

Perspectives

- Sensitive assays for *Toxoplasma*-specific DNA need further improvement and validation in clinical situations.
- Genotyping may be used to estimate the source of infection and markers of resistance in target genes coding for protein targets for antibiotics will provide information on possible drug resistance.

Acknowledgements

This work would never have been completed without the encouragement and continuous close collaboration from Birgitta Evengård throughout the development of the studies, data analysis and writing of the different papers.
Carl Erik Nord and Birgitta Evengård, Department of Bacteriology, at Karolinska University Hospital Huddinge, skilfully guided me through the rules and regulations of the Karolinska Institute and provided me with working space during my frequent stays in Stockholm.

These studies have been performed in collaboration with many European partners. The first study was perceived in the European Research Network on Congenital Toxoplasmosis, a European Union funded concerted action under the third framework programme. The EU funded programmes provided great opportunities for different European institutions to meet and discuss common problems in *T. gondii* diagnostics. I am particularly indebted to Philippe Thulliez, head of the *Toxoplasma* reference laboratory at the l'Insitut de Puericulture, Paris, for sharing his profound knowledge on *Toxoplasma* diagnosis in pregnant women, knowledge on antigen production in mice and new tests like the immuno sorbent agglutination assay and differentiated agglutination assay. Francois Peyron, Lyon, was always listening and imaginative and kept an open mind when we discussed shortcomings of different tests and possibilities for improvement. Klaus Hedman, Helsinki, invented the IgG-avidity assay, which proved a big step forward in our ability to determine the time of infection, and was a constant source of inspiration. Nicola Gargano had an intuition for selecting antigens for recombinant diagnostics assays that worked and was the first to develop an IgG-avidity assay based on recombinant antigens. Wilma Buffolano, Naples, has with great enthusiasm participated with her large database and well characterized samples from the central reference laboratory for pre- and neonatal infection for Campagnia Region, Italy.
The first European multicentre study using well defined antigens were analysed by Annie Robert from the Université Catholique de Louvain, Belgium, and twenty European centres contributed samples from pregnant women. I am indebted to Premyslaw Myjak who suggested new recombinant antigens to be tested for improved diagnostics and contributed with his cohort of patients.

Henrik Vedel Nielsen and Liss Wassmann, Laboratory of Parasitology, Statens Serum Institut, Copenhagen, overcame huge problems when the two-dimensional immunoblot was developed, which proved so useful in the end.

The European Multicentre Study on Congenital Toxoplasmosis, EMSCOT, supported by the 4th and 5th EU framework programme started out aiming at proving the benefit of pre- or neonatal screening in the largest prospective cohort study ever performed. It turned out otherwise and I am grateful for the many discussions and exchange of comments, which advanced our understanding of the questions still remaining concerning the benefit of pre-natal screening and I would like in particular to mention Arnold Pollak, Andrea Prusa and Michael Hayde (Vienna), Francois Peyron and Martine Wallon (Lyon), Philippe Thulliez and Stephane Romand , Paris, Hervé Pelloux, Grenobe, Margareth Paul, Poznan, Birgitta Evengård and Gunilla Malm, Stockholm, Nicole Ferret and Pierre Marty, Nice, Jacqueline Frank and Henri Dumont, Marseille, Enrico Semprini, Milan and Wilma Buffolano, Naples.

The data were analysed by Ruth Gilbert, Thalib Luukman, HK Tan and Luuk Graas from the Institute of Child Health, London, who did a tremendous job using advanced statistics and modelling and fought a continuous battle to get the results accepted and published when unexpected results appeared.

The collaboration with Oliver Liesenfeld, Berlin, Anne Naessens, Brussels, Maria Borobio, Sevilla, Ed Guy, Swansea, Valeria Meroni, Padua and Emi Spranzi from Diasorin testing a new, automated diagnostic system for IgG-avidty testing was a good experience in the problems of multicentre studies and requests for data to properly evaluate diagnostic systems.

Birgitta Evengård and Benjamin Edvinsson made me interested in *Toxoplasma* infections in immunosuppressed patients and Thomas Benfield, Copenhagen, generously gave me access to the Copenhagen HIV cohort.

I am grateful to Statens Serum Institut, my employer until 2003, which provided me with a great opportunity to develop and take part in the different multicentre studies funded by the European Union over the past fourteen years. My sincere thanks go to the staff at the Laboratory of Parasitology at Statens Serum Institut who always worked dedicated and skilfully with the different techniques used in the studies.

Layman summary

Infection with the parasite *T. gondii* is a problem in pregnant women, where the infection may be passed to the unborn child resulting in infection with or without symptoms. After infection with *T. gondii* the parasite remains dormant, but alive in the body for the rest of our lives, and may be reactivated and cause infection if our immune system fails like during a HIV-infection or if suppression occurs as after organ transplantations.

Diagnosis of infection with *T. gondii* is sometimes difficult because the usual pattern of antibody responses like the IgM-type antibodies usually only seen in acute infections, is found for many months after infection with *T.gondii*.

Common diagnostic assays were compared using sera from pregnant women with known time of infection, which allowed us to assess the performance of different assays according to the time since infection. It was found that a combination of two assays, the first detecting IgM-antibodies and the second measuring the maturation of the IgG response (IgG-avidity) provided the best information on the time of infection.

A study of diagnosis of *T. gondii* in newborn children showed that the IgM tests has a sensitivity as low as about 30% and almost completely failed to diagnose children born with *Toxoplasma*-infection acquired during the first half of pregnancy. This problem can partly be overcome by separating the antibody response in the child from the antibody response in the mother by a technique allowing the identification of the antibody response to many individual antigens (so called immunoblots).

Using *T. gondii* antigens produced in *E. coli* bacteria, recombinant antigens, and assays could be improved. Especially IgG-avidity assays using recombinant antigens showed superior performance and for other assays the background reactions were reduced, but the tests still need improvement to find all positive sera compared to tests using lysed, whole *T. gondii* parasites as antigen.

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