Prenatal diagnosis and treatment of congenital *Toxoplasma gondii* infections: an experimental study in rhesus monkeys

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Abstract

The efficacy of treatment in fetuses in whom congenital *Toxoplasma gondii* infection had been established has been investigated using rhesus monkeys as a model for humans. A polymerase chain reaction has been developed for the detection of *Toxoplasma gondii*. Using this polymerase chain reaction congenital infection can be established within 2 days of receiving an amniotic fluid sample. The polymerase chain reaction has subsequently been used to monitor the effect of treatment on fetal infection. The results show that early treatment with the combination of pyrimethamine and sulfadiazine was clearly effective in reducing the number of parasites in the infected fetus. The parasite was no longer detectable in the amniotic fluid 10 to 13 days after treatment was started. Spiramycin, on the other hand, has to be administered for at least 3 weeks to achieve the same effect. Moreover, pharmacokinetic studies revealed that spiramycin does not reach the brain. Pyrimethamine and sulfadiazine are able to pass the blood-brain barrier. Pyrimethamine appears to accumulate in the brain tissue and reaches concentrations which are also effective in vitro. © 1997 Elsevier Science Ireland Ltd.

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1. Introduction

When a mother has acquired primary *T. gondii* infection during pregnancy, the fetus may become infected as well. The risk of fetal infection is related to the gestational stage at the time of maternal infection. Studies from Desmonts and Couvreur [1] have shown that the transmission rate increases from 25% to 54% to 65% after infection in the first, second, and third trimester, respectively. More recently, studies of Wong and Remington revealed transmission rates of 15%, 30%, and 60% [2]. The severity of congenital infection also depends on the stage of pregnancy at which infection is acquired and the earlier it occurs, the more severe the infection [3]. *T. gondii* infection can cause non-specific symptoms such as intrauterine growth retardation, hepato-splenomegaly, purpura, and jaundice. A minority of congenitally infected fetuses will have severe manifestations such as hydrocephalus, microcephalus, chorioretinitis and intracranial calcifications and may even lead to death of the fetus.

Early diagnosis of fetal infection is important in order that an informed decision can be made about treatment or therapeutic abortion. However, prenatal diagnosis of congenital *T. gondii* infections was hampered by the lack of a reliable, sensitive and rapid diagnostic test. Serology, for instance, must rely solely on the presence of fetal IgM antibodies, because these antibodies do not cross the placental barrier, as do IgG antibodies. Antibody production, however, often fails or is delayed in the fetus with a still developing immune system. Congenital *T. gondii*
infection should therefore be proven by demonstrating the presence of the parasite but the diagnostic tests have their limitations; Microscopy is rather insensitive, detecting antigens is neither sensitive nor specific enough and tissue culture and mouse inoculation can be time consuming. In addition, not every laboratory is equipped with the facilities for tissue culture and animal research. Among the newer molecular techniques direct demonstration of the parasite by the polymerase chain reaction (PCR) showed the most promise. In 1989 when the experimental study described in the present paper was started the PCR on the BI-gene [4], a 35-fold repetitive gene which is specific for T. gondii appeared to be a sensitive and specific test for the detection of T. gondii in the amniotic fluid [5], cerebrospinal fluid and tissues of congenitally infected fetuses [6]. The PCR on the BI- gene, however, failed to detect T. gondii in amniotic fluid samples and blood samples from experimentally infected rhesus monkeys which were found positive by mouse inoculation [7]. Therefore, further investigations were necessary to enhance the sensitivity of the PCR and thus improve the prenatal diagnosis of congenital T. gondii infections.

Today PCR has proven to be of potential diagnostic value and it has become common practice to include the PCR for the antenatal diagnosis of congenital T. gondii infections. Currently, pregnant women suspected of having primary infection are tested serologically for the presence of specific IgM and IgG antibodies in the Netherlands. Treatment with spiramycin is initiated in most cases when the results obtained in serological testing are consistent with an infection acquired during pregnancy. Prenatal diagnosis of fetal infection is based on ultrasonography, amniocentesis and fetal blood sampling. Besides conventional parasitological tests (tissue culture and mouse inoculation) the PCR performed on amniotic fluid is an important technique to establish fetal infection as has been found by Hohlfeld et al. [8]. However, PCR is not yet a standardized test and because it is still under investigation the use of PCR should be restricted to specialist centres [9].

Congenital toxoplasmosis is treated with spiramycin, pyrimethamine and sulfadiazine, or a combination of these two drug regimens. The macrolide antibiotic spiramycin is mainly used in France. Spiramycin is given to pregnant women as soon as seroconversion has occurred. The antibiotic is known to be a safe drug during pregnancy. Beneficial effects of the antibiotic have been claimed. Placental infection is less frequently found in women treated with spiramycin [3,10]. A reduction in the incidence of congenitally infected infants born to treated versus untreated mothers is found as well [3]. Although these findings indicate that treatment with spiramycin reduces the risk of fetal infection, it is not known whether the antibiotic prevents transmission of infection or whether it has also a therapeutic effect on the infected fetus. The latter is probably not the case since spiramycin does not modify the pattern of clinically apparent infection in the fetus probably because spiramycin does not reach therapeutic concentrations in the fetus.

At present the synergistic combination of pyrimethamine and sulfadiazine is the treatment of choice for congenital toxoplasmosis and has been given to pregnant women for nearly 30 years. Several investigators report a significant reduction in the incidence of congenital infection after treatment with the pyrimethamine–sulfadiazine combination [11–13]. The drug regimen appears more effective than spiramycin in eradicating T. gondii parasites from the placenta [10]. Moreover, pyrimethamine–sulfadiazine treatment leads to a reduction in the number of severely affected babies and a shift to less severe and subclinical forms [14]. The effect of pyrimethamine–sulfadiazine has been claimed to be superior to spiramycin but no prospective, randomized trials have been conducted. Therefore, more research was needed on treatment of fetuses with proven congenital T. gondii infections.

1.1. Aim of the study

Before introduction of the PCR test, prenatal diagnosis of congenital T. gondii infections was unreliable and so a woman who acquired a primary T. gondii infection during pregnancy was forced to decide between treatment or terminating her pregnancy without knowing whether the fetus was infected. Moreover, clinicians could not guarantee that treatment would be effective nor could they be reasonably sure that the fetus was infected in which case treatment would merely be preventative.

Therapeutic abortion in The Netherlands is legally permissible until 24 weeks gestation, when the rate of transmission to the fetus is ≤ 15%. Thus, the choice of termination of pregnancy would lead to a high number of unnecessary abortions. It was therefore important to improve the prenatal detection of T. gondii infections and also to conduct prospective, randomised studies on the effectiveness of treatment of the infected fetus.

The following items were studied:

1. How can fetal infection with T. gondii be established when there is serological evidence of primary infection in the mother?
2. What is the effect of chemotherapeutic treatment of the mother during gestation in the congenitally infected fetus?

2. Results of the study and general discussion

A new diagnostic test was developed to improve the prenatal diagnosis of congenital T. gondii infections. The PCR method was chosen because this technique has already proven its applicability as a diagnostic test in a
variety of other microbiological infections. The principle of a diagnostic PCR is in vitro amplification of specific target DNA sequences on the genome of interest (in this case *T. gondii*). This way, it is theoretically possible to detect a single molecule within the abundance of host DNA.

Initially, the PCR on the BI-gene described by Burg et al. [4] was chosen. The applicability of this PCR was tested on clinical samples in which the presence of *T. gondii* was proven by conventional diagnostic techniques, such as mouse inoculation, tissue culture, and/or microscopy [6]. The PCR on the BI-gene, however, failed to detect *T. gondii* in amniotic fluid samples and blood samples from experimentally infected rhesus monkeys which were found positive by mouse inoculation. To improve the sensitivity of the PCR, a nested PCR was developed with the small subunit ribosomal DNA gene (ss rDNA) as target [15]. A nested PCR is characterised by performing two PCR’s subsequently, using the amplified product of the first reaction as the target for the second reaction. For the development of the nested PCR the complete nucleotide sequence of the ss rDNA was elucidated. The rDNA gene was chosen because it contains several *T. gondii*-specific sequences. In addition, the gene product, the ribosomal RNA, is present in about 10,000 copies per cell (or per parasite) and can also be used as target in PCR [15].

The nested PCR on the rDNA gene appeared to be more sensitive than mouse inoculation, and fulfilled the qualities of a diagnostic test for the prenatal detection of *T. gondii*. It was possible to demonstrate the presence of *T. gondii* within two days of receiving the clinical sample by using the PCR. Using the conventional mouse inoculation, a test result was obtained one to three weeks later.

The results show that the PCR is a suitable test for the direct demonstration of *T. gondii* in the congenitally infected fetus. With the exception of one amniotic fluid sample which was not available for PCR, 6 of 7 proven fetal infections were found positive by PCR in the rhesus monkeys. PCR should be added as a diagnostic tool or it may even replace the conventional techniques. Amniotic fluid samples appeared to be the clinical sample of first choice for the prenatal diagnosis of *T. gondii* infection [7].

A recent study among pregnant women with a primary *T. gondii* infection supports our findings that a PCR on amniotic fluid performed better than conventional parasitological methods for the prenatal detection of the parasite [8]. The investigators demonstrated congenital infection in all of the 34 cases, in which transmission of infection had occurred, by PCR. Follow-up testing, using the conventional methods, confirmed the presence of congenital toxoplasmosis [8].

The PCR is currently used as an additional diagnostic tool for the prenatal diagnosis of congenital *T. gondii* infection at the department of Medical Microbiology of the Academic Hospital ‘‘St. Radboud’’ in Nijmegen.

After the investigations on the improvement of a diagnostic technique for the detection of *T. gondii* infections, the study focused on the effect of treatment in the infected fetus when the drugs are given to the mother. These experiments were carried out in rhesus monkeys (*Macaca mulatta*) for logistic and practical reasons. Moreover, transmission of infection was expected to be similar for rhesus monkeys and humans because they both possess a placenta of the hemochorial type. The animal experiments were performed after accreditation by the ethical committee of the Faculty of Medical Science, according to the Law on Animal Experimentation in The Netherlands.

The rhesus monkey seemed a suitable animal on which to perform these investigations. Former studies have shown that this monkey served well as a model for human congenital *T. gondii* infections [7]. The frequencies of transmission of infection which were found in the rhesus monkey after maternal infection in the second and third trimester of gestation equal those observed in humans.

The traditional therapy for congenital *T. gondii* infections is pyrimethamine in combination with a sulfonamide, usually sulfadiazone. Spiramycin is less active than the pyrimethamine–sulfadiazine combination, but it is capable of inhibiting the growth of free and intracellular parasites. The studies focused on these two drug regimens since it was still unclear whether or not these anti-*Toxoplasma* drugs reach therapeutic concentrations in already infected fetuses. The dosage regimens for the drugs was established by pharmacokinetic studies in the rhesus monkeys, since such data were not available for this animal species. Rhesus monkeys were infected in the second trimester of pregnancy. Treatment with anti-*Toxoplasma* drugs was started immediately after fetal infection had been proven and continued throughout pregnancy. Transmission of infection occurred in 5 of 8 monkeys in the group treated with spiramycin and in 6 of 10 monkeys in the group treated with pyrimethamine and sulfadiazone. The effect of treatment on fetal infection was monitored by PCR. It appeared that spiramycin reduced the number of parasites in the amniotic fluid to undetectable levels within a period of three weeks [16]. The pharmacokinetic studies revealed that spiramycin accumulated in the tissues of mother and fetus, but spiramycin was not found in the brain tissue [16,17]. In comparison with spiramycin pyrimethamine in combination with sulfadiazone is more effective on congenital *T. gondii* infection. *T. gondii* parasites were no longer found in the amniotic fluid after 10 to 13 days of treatment with pyrimethamine and sulfadiazone [18]. In contrast with spiramycin, both pyrimethamine and sulfadiazone were able to cross the blood–brain barrier. Moreover, pyrimethamine was found to accumulate in the brain tissue and reaches therapeutic levels [19].

The pharmacokinetic studies of sulfadiazone in the rhesus monkey revealed the presence of 5 metabolites. Three of these metabolites have not (yet) been found in
humans [19]. The metabolites appeared to be present not only in the urine, but in plasma as well. It was not known whether these metabolites possessed anti-"Toxoplasma" activity and thus whether they contributed to the efficacy of treatment. Therefore, anti-"Toxoplasma" activity of these metabolites has been studied in vitro [20]. Except for N4-acetyl-sulfadiazine, all metabolites possess anti-"Toxoplasma" activity in vitro.

The finding of metabolites of sulfadiazine in the rhesus monkey which are not present in humans raises the question whether these metabolites contribute to the efficacy of treatment. Pharmacokinetic studies in the rhesus monkey revealed that the metabolites 5-OH-glucuronide-sulfadiazine and 5-OH-sulphate-sulfadiazine do not contribute to the efficacy of treatment, since effective serum concentrations are not reached [19]. The contribution of two other metabolites, 5-OH-sulfadiazine and 4-OH-sulfadiazine, to the efficacy of treatment is rather low, since they appear to be present in small proportions in the plasma of the rhesus monkeys [19]. All together, the proportion of active sulfa-compounds in serum of humans appears to be similar to what has been found in rhesus monkeys. These arguments suggest that extrapolation of the findings in the rhesus monkey to the human situation is justified.

The improvement of the prenatal detection of congenital *T. gondii* infections and the effect of treatment in the infected fetus logically leads to the question whether there is a place for a routine serological screening on a primary infection during pregnancy or not. Acute *T. gondii* infection in pregnant women almost always goes unrecognized and will continue to be missed unless a systematic antenatal toxoplasmosis screening programme is initiated. However, before any screening programme can be introduced, the benefits and risks of such a programme have to be thoroughly evaluated. It should also be taken into account that what seems appropriate for one country is not always appropriate for another, since the frequency of infection differs per geographic area. In fact, the following factors need to be investigated before considering such a screening programme:

1. The prevalence of immunity to toxoplasmosis in women of childbearing age.
2. The incidence of primary infection in pregnancy
3. The rate of transmission to the fetus
4. The predictive value (sensitivity, specificity, and reproducibility) of a serological screening test to diagnose a primary infection in the pregnant woman.
5. The reliability of diagnosing infection of the fetus.
6. The efficacy of treatment for the fetus, when the drugs are given to the mother.

The items 5 and 6 are the topics of the foregoing studies, and they are both conditions for introduction of a serological screening programme. Some remarks are made with regard to these items.

With PCR a test result can be obtained within two days of receiving the clinical sample. In addition, PCR is suitable for monitoring the effect of treatment by analysis of amniotic fluid before and after initiation of therapy. Thanks to the considerable gain of time a pregnant woman, suspected of a primary *T. gondii* infection, can await the test result of the PCR before taking a well considered decision.

Based on the findings in humans that treatment with spiramycin reduces the number of infected offspring, treatment with spiramycin might be considered for primary prevention (i.e. prevention of transmission of infection) when transmission of infection has not (yet) occurred. The findings in rhesus monkeys, however, do not support the use of spiramycin to prevent transmission of infection. Although spiramycin accumulates in the placenta, it never reaches the high concentrations that are reported to be necessary to inhibit *T. gondii* in vitro. Pyrimethamine and sulfadiazine on the other hand, do reach effective serum and tissue concentrations. In addition, with pyrimethamine and sulfadiazine it takes 10 to 13 days to reduce the number of parasites to undetectable levels in the amniotic fluid. It is therefore expected that it will take about the same time to reduce the parasite in the blood circulation of the mother. Based on these findings, one course of 4 weeks of pyrimethamine with sulfadiazine for prevention of transmission of infection is preferred over that with spiramycin. This regimen also corresponds with that for healthy neonates, born to women with a proven primary infection during pregnancy, but in whom congenital infection is not definitely proven.

Treatment with spiramycin for secondary prevention (i.e. after transmission of infection has occurred) is only partially effective. It appears from the results that spiramycin should be administered for at least three weeks to reduce the number of parasites in the amniotic fluid. Moreover, spiramycin does not reach the brain. Spiramycin will not be the treatment of choice for secondary prevention, since cerebral infection is the most severe consequence of *T. gondii* infection in the fetus. The combination of pyrimethamine and sulfadiazine is preferred over spiramycin to prevent symptoms in the fetus when antenatal infection is proved. The results indicate that treatment with pyrimethamine–sulfadiazine efficiently reduces the number of parasites in the fetus; as mentioned before no parasites were detected in the amniotic fluid 10 to 13 days after initiation of therapy. In contrast with spiramycin, both pyrimethamine and sulfadiazine reach the brain tissue. Pyrimethamine even accumulates in the brain.

Our studies thus demonstrate that treatment with pyrimethamine and sulfadiazine is effective in reducing the number of parasites in the infected rhesus monkey fetus. The results have been found under experimental conditions, in which rhesus monkeys have been treated for congenital *T. gondii* infections with a dosage regimen that
is also used in humans. Extrapolation of the results to the human situation, however, should be interpreted with care, since the pharmacokinetics of pyrimethamine and sulfadiazine in rhesus monkeys differ in some ways from the pharmacokinetics in humans. For instance, the serum elimination half lives of both pyrimethamine and sulfadiazine are shorter in rhesus monkeys than in humans [18]. However, whether it is valid to extrapolate the effect of a treatment in monkeys to the human situation depends on the serum and tissue concentrations which are achieved and the period of time during which these effective concentrations are present.

Effective serum concentrations of pyrimethamine and sulfadiazine as found in rhesus monkeys appear also to be present in humans. In addition, the period of time during which these effective serum concentrations are found in humans is at least as long as or even longer than those found in the rhesus monkeys [21–23].

It must be realized that the results described here were obtained under experimental conditions in which the time of maternal infection was exactly known, prenatal detection of the parasite was frequently performed to monitor fetal infection, and treatment was started early after fetal infection. This is at variance with the natural situation, in which maternal infection is often not recognized because the infection is mostly asymptomatic and serological screening is not routinely performed. As a consequence, a majority of the fetal infections will remain unrecognized or diagnosed too late in order to guarantee that treatment still will be effective.

The findings plead for early treatment of the infected fetus and thus plead, indirectly, for introduction of a screening programme for a primary T. gondii infection during pregnancy. Rapid diagnosis of fetal infection is possible with the development of a diagnostic PCR for T. gondii. This creates the possibility to start treatment early after fetal infection has occurred. Although congenital toxoplasmosis may be a preventable disease, the effect of primary prevention is expected to be not sufficient as it is difficult to change the behavior of pregnant women or women who are attempting to conceive with regard to taking preventive measures. This, the improvement of the prenatal detection of T. gondii and the knowledge that pyrimethamine and sulfadiazine are effective in the infected fetus are three arguments in favor of reconsidering the need and practical implementations of introducing a serological screening programme in the Netherlands. As long as vaccination is not yet feasible, follow up in serological negative women, starting before or early after conception, has to be reconsidered, despite the fact that it is recognized not to be cost-effective.

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