Eur J Clin Microbiol Infect Dis (1998) 17:853–858

Article

Follow-Up of Infants with Congenital Toxoplasmosis Detected by Polymerase Chain Reaction Analysis of Amniotic Fluid

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Abstract This study was conducted to assess the validity of performing the polymerase chain reaction (PCR) on amniotic fluid for detecting fetal *Toxoplasma* infection. The primary endpoint was the outcome of the infant at 1 year of age. A prospective, consecutive study was performed in 49 infants born to mothers with primary *Toxoplasma* infection during pregnancy. PCR determinations of *Toxoplasma gondii* DNA in amniotic fluid were carried out as part of their prenatal management. Infants were examined at birth, and at 1, 3, 6, 9, and 12 months of age. Nine of 11 infants from pregnancies with positive PCR results proved to be infected based on follow-up serological investigations conducted during the first year of life. Two fetal deaths occurred. All 38 infants with negative PCR results remained uninfected at 1 year of age, irrespective of whether their mothers had received treatment with sulfadiazine/pyrimethamine or spiramycin alone. Psychomotor development was normal in all infants. This follow-up study confirms that PCR performed on amniotic fluid is a useful method for identification or exclusion of fetal *Toxoplasma* infection. Treatment of infected pregnant women and – in the event of a positive PCR result – subsequent treatment of their infants is associated with a favorable outcome.

Introduction

The clinical spectrum of congenital *Toxoplasma* infection varies from an apparent disorder at birth to a subclinical infection with a high risk for developing retinochoroiditis and/or neurological complications later in life [1–4]. In order to provide appropriate treatment for all infants at risk, definitive diagnosis of congenital infection at birth is mandatory [5, 6].

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Maternal follow-up serological tests during pregnancy allow only an indirect estimation of the risk of fetal infection [5, 7]. In contrast, the direct detection of *Toxoplasma* DNA in amniotic fluid by means of the polymerase chain reaction (PCR) is currently viewed as the most sensitive, specific, and rapid method for diagnosis of fetal infection [8–11]. However, the assumption that *Toxoplasma*-positive amniotic fluid denotes and a negative PCR result contradicts fetal infection at the time of amniocentesis has never been validated by means of follow-up data obtained on infants from such pregnancies.

In this study we report the results of PCR performed on amniotic fluid obtained by amniocentesis from 48 women with primary *Toxoplasma* infection during pregnancy and the corresponding follow-up data of their infants during the first year of life with regard to serological and clinical outcome, including fundoscopy, assessment of psychomotor development, and cranial ultrasound examination.

Patients and Methods

Primary maternal *Toxoplasma* infections during pregnancy were classified either as definite infection if seroconversion had occurred, or as probable infection if a significant (at least 4-fold) rise in IgG titers had occurred with the simultanous presence of IgM antibodies. Alternatively, infection was considered probable if a high IgG titer and the presence of IgM were observed in the second half of pregnancy [6]. A high IgM titer most likely reflects a recent infection. Lower levels of IgM antibodies may persist over months or even years.

The diagnosis of fetal infection was based on the demonstration of *Toxoplasma* DNA in amniotic fluid with or without persistent ultrasound findings such as ventricular dilatation and/or echogenic intracerebral lesions. A congenital infection was defined by rising or persistent IgG titers within the first 12 months of life, regardless whether IgM was positive within the first months of life. Congenital infections were divided into clinical infections in which obvious symptoms such as hydrocephalus, intracerebral calcifications, or retinochoroiditis were present, or subclinical infections in which clinical signs were absent [6].

In the Austrian Toxoplasmosis Prevention Programme, it is recommended that a serological test be performed three times during pregnancy: in the first, second, and third trimesters. If one of the tests reveals definite or probable primary maternal infection (see above), treatment is initiated [12]. In short, treatment consists of spiramycin (3 g/day) prior to week 16 of gestation. From week 16 onwards until delivery, combination treatment with sulfadiazine (3 g/day), pyrimethamine (25 mg/day), and folinic acid (5 mg 2 times per week) is administered for 4-week periods alternating with 4-week periods in which spiramycin is administered.

A total of 48 pregnant women (1 twin pregnancy) with definite or probable primary infection underwent amniocentesis during March 1992 and March 1995. None of the women had HIV infection or was receiving steroids or immunosuppressive drug treatment (Table 1). In 13 cases amniotic fluid samples were obtained outside of Vienna, sent to this laboratory for PCR analysis, and processed immediately. After amniocentesis all women received treatment during pregnancy until delivery. All infants from pregnancies with positive PCR results were treated after birth until the end of the first year of life. The infants were born between April 1992 and April 1995.

Infants were monitored serologically at regular intervals by means of the Sabin-Feldman dye test [13] and measurement of specific IgM [14]. The initial sample was obtained from cord or venous blood during week 1 of life, while follow-up samples were obtained at ages 1, 3, 6, 9, and 12 months. The individual dye test titers of the infants were compared with the physiologic curve of decay of transplacentally transmitted maternal IgG antibodies against *Toxoplasma gondii* used as the reference curve [5]. A child was classified as uninfected if the individual titer did not exceed one standard deviation above the mean curve at a given time postpartum [5]. Physical examination, body measurements, and assessment of psychomotor development were carried out on the same dates. Cranial ultrasound examinations to detect intracerebral calcifications or ventricular enlargement were performed at ages 1–3 months and 6–9 months. Fundoscopy to detect retinochoroiditis was performed in the first and last quarter of the first year.

If an infant was considered infected (see definitions above), treatment was initiated [5, 7]. Newborn infants with apparent disease received sulfadiazine (85 mg/kg/day), pyrimethamine (1 mg/kg/day), and folinic acid (5 mg 2 times/week) over a period of 6 months, followed by alternating cycles of spiramycin (100 mg/kg/day for 6 weeks) and sulfadiazine/pyrimethamine (4 weeks) until the end of the first year of life. Infants with subclinical infection received 4-week cycles of spiramycin alternated with 4-week cycles of sulfadiazine/pyrimethamine until age 1 year.

A total blood count was obtained every other week. Except for one patient (patient 4, see below) with transient mild anemia, all infants tolerated the treatment without obvious side effects.

Table 1 Characteristics of 48 patients with *Toxoplasma* infection during pregnancy (1 twin pregnancy included)

Characteristic	PCR result	
	Negative ($n=38$)	Positive ($n=11$)
Maternal age in years (mean ± SD)	28.5 ± 6.2	25.2 ± 4.1
Maternal serology[a]		
No. who seroconverted	10 (0/5/5)	9 (1/5/3)
No. with rising or primary high titer, positive IgM	28 (4/20/4)	2 (0/2/0)
Amniocentesis		
Gestational age in weeks (mean ± SD)	22.9 ± 6.3	25.6 ± 3.6
Duration of treatment before amniocentesis[b]		
No treatment (no. of patients)	8	5
Up to 4 weeks (no. of patients)	19	4
4 to 11 weeks (no. of patients)	11	2
Duration in weeks (mean ± SD)	3.4 ± 3.4	1.8 ± 3.2
Treatment after amniocentesis (until delivery)[b]		
Spiramycin only (no.)	11	1
Sulfadiazine + pyrimethamine/spiramycin (no.)	27	10
Birth		
Gestational age in weeks (mean ± SD)	39.2 ± 2.3	37.0 ± 5.3
Birthweight in grams (mean ± SD)	3255 ± 799	3102 ± 509[c]

[a] Details, see Patients and Methods
Numbers in parentheses refer to time of diagnosis of primary *Toxoplasma* infection in the first, second, and third trimester, respectively
[b] Treatment depended on gestational age; for details, see Patients and Methods
[c] Birthweights of patients 1 and 2 (fetal death) lacking

Serological Investigations. Confirmatory serological tests were performed on both maternal and infant follow-up sera. An immunoabsorbent agglutination assay (Toxo-ISAGA; bioMérieux, France) [14] was used for determination of *Toxoplasma*-specific IgM. Its high sensitivity is especially useful in the diagnosis of congenital *Toxoplasma* infection. For IgA detection the Eti-Toxok-A kit (DiaSorin; Biomedica, Italy) [15] was used. The Sabin Feldman dye test was carried out as described previously [14].

Polymerase Chain Reaction. Approximately 10 ml of amniotic fluid was centrifuged to obtain cellular material. Cells were treated with lysis buffer (NaOH 10 mM, Tween-20 0.5%, Nonidet-40 0.5%), heated at 95 °C, chilled, and centrifuged. The supernatant was used for PCR. Two different regions of the B-1 gene were amplified: a region of 120 base pairs (bp) and a fragment of 180 bp. Reagent blanks were processed throughout all procedures as negative controls to detect contamination. DNA from *Toxoplasma* trophozoites was used as positive control. In addition, amplification of artificial DNA was used as internal positive control, resulting in a fragment of 340 bp [16]. The likelihood of contamination was reduced by performing the PCR with uracil-N-glycosylase (UNG) and deoxyuridine triphosphate instead of deoxythymidine triphosphate (UNG Carry-over Prevention; Perkin Elmer, USA) [17]. Oligonucleotides were from Genset, France, reaction mix and *Taq* polymerase from Perkin Elmer, USA.

The PCR products were separated by 8% nondenaturating acrylamide gel electrophoresis, stained with ethidium-bromide, and visualized under UV illumination.

Results

Negative Polymerase Chain Reaction Results. Thirty-eight of 49 (77.6%) amniotic fluid specimens tested (1 twin pregnancy included) were negative in the PCR. Twenty-eight (73.7%) pregnancies were classified as probable infection and ten (26.3%) as proven infection based on seroconversion (Table 1). Thirty women received treatment up to 11 weeks prior to amniocentesis, while eight women had no treatment at the time of amniocentesis but were treated immediately thereafter.

Treatment was continued until delivery in all women, but the drug regimens differed. Eleven (28.9%) women received spiramycin as monotherapy, and the remaining 27 (71.1%, twin pregnancy included) were treated with sulfadiazine/pyrimethamine according to the program recommendations. Fetal sonography was normal in all cases except for one with hydrocephalus unrelated to toxoplasmosis (patient 12, details see below).

Congenital infection was excluded in all infants at follow-up, irrespective of whether their mothers had received sulfadiazine/pyrimethamine or spiramycin alone: Cord blood samples and all blood samples drawn during the 1-year follow-up period were IgM-negative. Transplacentally transmitted IgG antibodies became negative within 6–12 months in all infants. All except patient 12 (details see below) presented as clinically normal. Cranial sonography and fundoscopy were also normal.

In patient 12 maternal *Toxoplasma*-specific IgG antibodies rose significantly between weeks 14 and 28 of gestation. Therapy was initiated at week 28. Hydrocephalus, intracranial calcifications, and microphthalmus were detected by fetal sonography. Amniotic fluid PCR was negative at week 31. Retrospectively collected data revealed a low IgG titer (1:16) in a previous pregnancy in 1984. The infant was born prematurely at week 33, weighing 1670 g. It died 1 month later because of severe sepsis and intraventricular hemorrhage. Congenital toxoplasmosis was excluded at autopsy. Histological examinations of the brain and placenta were negative.

Positive Polymerase Chain Reaction Results. Eleven (22.4%) amniotic fluid samples tested positive for DNA from *Toxoplasma gondii*. Nine (81.8%) pregnant women experienced seroconversion and two (18.2%) IgM positivity with a significant rise in dye test titers (Table 2). Five women were untreated at the time of amniocentesis. In the remaining four, initiation of treatment preceded amniocentesis by 1 week (Table 1). One abortion was performed at week 25 (patient 2) and one fetal death occurred at week 29 (patient 1). All women except patient 2 received the combination therapy consisting of sulfadiazine/pyrimethamine throughout pregnancy until delivery.

One infant (patient 10) was delivered prematurely at week 35 of gestation but had no further problems in the neonatal period. In two of nine liveborns (patients 3 and 6) IgM was positive in cord blood; interestingly, the only patient tested for IgA in cord blood was positive despite negative IgM (patient 10). All infants were apparently healthy at birth except for one infant (patient 3), who had hydrocephalus and another one with ventriculomegaly (patient 4). Ventricular shunting was not necessary in either case, and head circumferences remained within normal limits. In addition, one of these two infants had a retinal scar (patient 3). All infants with or without clinical evidence of congenital toxoplasmosis (except patient 10) were treated throughout the first year of life. At follow-up through age 1 year, all the infants, including the two patients with cerebral manifestations of the disease, exhibited normal physical and psychomotor development.

Details of Cases in which Polymerase Chain Reaction was Positive. In patient 1 amniocentesis was performed at week 25 of gestation because of fetal hydrocephalus of unknown etiology. PCR for *Toxoplasma gondii* was positive. Serological tests performed on maternal sera revealed seroconversion at week 28. Fetal death occurred at week 29 of gestation. Histological examination of the placenta and brain revealed severe infection with *Toxoplasma gondii*. The official

Table 2 Testing and treatment of 11 women with positive PCR result during pregnancy

Patient no.	Maternal serological status	Treatment	Gestational age at birth (weeks)	Birthweight (g)	Gender	Cord IgM	Fundoscopy	Cranial ultrasound	Psychomotor development
1[a]	SC	S+D	29	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
2[a]	SC	S	25	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
3	SC	S+D/S	40	3200	f	pos	retinal scar	hydrocephalus	normal
4	SC	S+D	37	2790	f	neg	normal	ventriculomegaly	normal
5	SC	S+D/S	40	2960	f	neg	normal	normal	normal
6	SC	S+D/S	40	3350	m	pos	normal	normal	normal
7	SC	S+D/S	40	3680	f	neg	normal	normal	normal
8	PHT	S+D/S	40	3300	f	neg	normal	normal	normal
9	SC	S+D/S	39	3600	f	neg	normal	normal	normal
10	SC	S+D/S	35	2110	f	IgA pos[b]	normal	normal	normal
11	PHT	S+D/S	40	2990	m	neg	normal	normal	normal
			25.6±3.6[c]	3102±509[c]					

[a] Fetal death, no further data available
[b] IgM was negative, IgA was positive
[c] Mean±SD

SC, seroconversion (=definite infection); S+D, sulfadiazine+pyrimethamine; n.a., not available; S, spiramycin; pos, positive; neg, negative; PHT, rising or primary high titer and positive IgM

screening recommendations were not followed properly in this case. The first test at week 10 was negative, but no follow-up serological test was performed in the second trimester of pregnancy, when seroconversion must have occurred.

Patient 2 seroconverted between weeks 9 and 11 of gestation and was treated with spiramycin from week 12 to week 18. Fetal ultrasound showed hepatomegaly and ascites. PCR was positive at week 22, and the pregnancy was terminated at week 25.

In patient 3 seroconversion must have occurred between weeks 10 and 27, but screening recommendations were not followed properly. Fetal hydrocephalus was observed at week 27 and treatment with sulfadiazine/pyrimethamine was started. Delivery occurred at term, and birthweight was 3200 g. Cord blood IgM was negative. Hydrocephalus was confirmed postpartum by ultrasound, and a retinal scar was discovered. The infant was treated immediately postpartum. Ventricular size declined, and shunt implantation could be avoided. PCR performed on cerebrospinal fluid was negative. Psychomotor development of the infant was normal at follow-up examinations.

Patient 4 seroconverted between weeks 12 and 25, but no treatment was given, because she was abroad. At week 31 fetal hydrocephalus was diagnosed by ultrasound examination. PCR performed on amniotic fluid was positive. Treatment was started at week 31 and continued until delivery. A second amniocentesis at week 34 revealed persistence of the parasite in amniotic fluid, despite treatment. The infant was born at week 37, weighing 2790 g. Moderate ventricular enlargement was noted, which normalized during the first year of life. Fundoscopy remained normal. Psychomotor development was normal at 1 year of age.

Patients 5, 6, 7, 9, and 10 seroconverted during pregnancy. Patients 8 and 11 exhibited a significant rise in dye test titers along with positive IgM titers in first and second tests. PCR performed on amniotic fluid was positive between weeks 26 and 30. In all cases drug therapy was administered until delivery, which occurred at a mean gestational age of 39.1 weeks ±1.7 weeks. The mean birthweight was 3140 g ±490 g. Since all infants appeared normal and healthy, they were classified as having subclinical congenital toxoplasmosis. Dye test titers remained positive at 12 months. Cognitive and motor development have been normal thus far.

In patient 10 treatment with sulfadiazine/pyrimethamine was given after seroconversion. The test for IgA, not performed routinely, was positive despite a negative IgM result. The infant appeared healthy after birth and at follow-up, but her mother refused further therapy despite detailed counseling.

Discussion

This is the first report of a prospective 1-year follow-up study of infants from pregnancies in which PCR was performed on amniotic fluid for the purpose of diagnosing fetal *Toxoplasma* infection. Previous studies [8, 9, 11] did not include long-term follow-up of affected infants. It has been shown previously [9, 11] that PCR performed on amniotic fluid is useful for both diagnosis and exclusion of fetal *Toxoplasma* infection. Our study confirms this result by defining infant outcome at 1 year of age as the endpoint for evaluating the benefit of PCR. All 11 fetuses for whom PCR of amniotic fluid was positive for *Toxoplasma* DNA were found to be congenitally infected. Of nine liveborns, two had classic symptoms such as hydrocephalus and retinochoroiditis.

The remaining seven were apparently healthy and had no clinical signs or symptoms of intrauterine infection.

Thus, PCR on amniotic fluid can be used to diagnose *Toxoplasma* infection of the fetus. In case of fetal infection, in utero treatment with sulfadiazine/pyrimethamine is recommended until delivery, followed by postnatal treatment of the infant during the first year of life. Many infants with subclinical infection would have been missed in the pre-PCR era, since only 60% express IgM and 75% are positive for IgA in cord blood [18]. When PCR on amniotic fluid was negative, indicating an unaffected fetus, treatment with spiramycin alone was sufficient to prevent delayed parasite transfer in all cases [11].

All nine infected infants remained IgG positive throughout the first year of life. In all cases, including those with abnormal brain ultrasound scan, physical and psychomotor development was normal at the end of the first year of life. We conclude that vigorous treatment of infected pregnant women by means of combination therapy with sulfadiazine/pyrimethamine followed by consequent postnatal therapy during the first year of life results in a favorable outcome; however, further long-term observations over many more years are necessary to exclude the occurrence of retinal lesions or neurologic abnormalities. Determining the duration of treatment of infected children is difficult: since dye test-titers are modified by treatment, they may fall rapidly during the first year but relapse thereafter, when therapy is discontinued [5].

Furthermore, our data suggest that fetuses with negative amniotic fluid PCR results remain uninfected if maternal treatment with spiramycin is provided throughout the remaining pregnancy. We did not observe any difference in fetal outcome if mothers with negative PCR results were treated with sulfadiazine/pyrimethamine or with spiramycin alone. Spiramycin is believed to prevent parasite transmission, since the drug accumulates in the placenta [19]. Sulfadiazine and pyrimethamine act synergistically and cross the placental barrier, thus permitting in utero treatment of infected infants and preventing further progression of fetal infection. Under these circumstances, a negative PCR result makes treatment during the first months of life unnecessary, since most authors recommend treatment if the status of infection of the newborn infant is unclear [7].

Seroconversion during pregnancy indicates a high risk of fetal infection [5]. In 82% of the cases in which PCR results were positive, maternal seroconversion had occurred. In two cases the outcome was fetal death in the second trimester. Only 18% of our patients with a positive PCR result belong to the group with probable maternal infection (Table 1). In countries such as France and Austria, where a well-established serological screening program for detection of *Toxoplasma* infection during pregnancy is in place, early treatment of maternal infection seems to be effective in preventing and/or treating fetal infection. The shorter the period between serological tests in seronegative pregnant women, the more effective the screening. In Austria two test repetitions in primary seronegative pregnant women are recommended [12]. Prolongation of test intervals or omission of serological follow-up tests increases the risk for fetal infection to occur undetected, such as in patient 1, 3, and 4.

Prenatal diagnosis represents a crucial component in counseling a pregnant woman with acute toxoplasmosis. A negative PCR result may provide relief from anxiety and motivate women to receive further treatment. Because the risk of amniocentesis is not negligible, even in experienced hands [20], this procedure should be offered only to pregnant women with serologically proven or probable primary *Toxoplasma* infection. Positive PCR on amniotic fluid confirms fetal infection and is a clear indication for combination treatment with sulfadiazine/pyrimethamine until delivery, treatment, and follow-up of the infant.

In conclusion, this study demonstrates the value of PCR for detecting fetal *Toxoplasma* infection. A positive test result is an indication for combination therapy consisting of sulfadiazine/pyrimethamine, whereas a negative result allows for treatment with spiramycin alone in order to prevent delayed transmission of the parasite. The validity of this management has been confirmed by 1-year follow-up studies.

Acknowledgement The skillful technical assistance of Barbara Rudan is gratefully appreciated. This study was supported by the Austrian National Bank (Jubiläumsfondprojekt Nr. 4327) and the "Verein zur Förderung der Forschung in der Neonatologie und Kinderintensivmedizin".

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