Urine Sample Used for Congenital Toxoplasmosis Diagnosis by PCR

ISABEL FUENTES,1* MERCEDES RODRIGUEZ,1 CARLOS J. DOMINGO,1 FERNANDO DEL CASTILLO,2 TERESA JUNCOSA,3 AND JORGE ALVAR1

Servicio de Parasitología, Centro Nacional de Microbiología, Instituto de Salud Carlos III,1 and Departamento de Pediatría, Hospital Infantil La Paz,2 Madrid, and Servicio de Microbiología, Hospital San Juan de Dios, Barcelona,3 Spain

Received 1 February 1996/Returned for modification 5 March 1996/Accepted 8 July 1996

The diagnosis of toxoplasmosis in congenitally infected infants can be difficult; serology is unreliable, and diagnosis must be based on the combination of symptomatology and direct demonstration of the parasite. Four infants suspected of having Toxoplasma gondii infection were studied by serological analysis, tissue culture, and PCR determination. T. gondii was isolated from the urine of one patient. The parasite was detected by PCR in the blood and cerebrospinal fluid of three infants and in the urine in all patients. Because nested PCR proved to be a sensitive, relatively rapid, and specific method and because it can be applied to a variety of different clinical samples, PCR can be a valuable technique for the identification of T. gondii infections in children. The present study indicates that PCR examination of urine, a fluid never before used for diagnosis in this age group, may be valuable in diagnosing cases of congenital toxoplasmosis.

MATERIALS AND METHODS

Case report 1. A 27-day-old underweight girl presented with hydrocephaly, ischemic lesions in the cerebral cortex, and detachment and vitreous blur of the retina. The mother had not been tested for toxoplasmosis during pregnancy.

Case report 2. A 6-day-old underweight girl presented with intracranial calcifications. The mother showed immunoglobulin M (IgM) antibodies specific for T. gondii in the third month of pregnancy.

Case report 3. A 24-day-old girl presented with intracranial calcifications. The mother presented with specific IgM antibodies during pregnancy.

Case report 4. A 14-day-old girl presented with neurologic symptoms and hydrocephaly. The mother was previously seronegative and had not been tested during pregnancy, but had specific IgG antibodies after childbirth.

Serology. T. gondii-specific antibodies were detected by several methods.

(i) Indirect immunofluorescence test. The indirect immunofluorescence test for anti-toxoplasma-specific IgG antibodies was performed as described previously (31).

(ii) Avidity ELISA of IgG. The avidity of T. gondii-specific IgG was measured by a protein-denaturing enzyme-linked immunosorbent assay (ELISA) as described previously (15, 21). Briefly, duplicate samples of serially diluted sera were introduced in microtiter wells coated with T. gondii antigen. After incubation, one well of each pair was washed with phosphate-buffered saline (PBS) and the other well was washed with a protein denaturant (6 M urea). The proportion of residual to total antigen-bound IgG was quantified immunoenzymatically. The ratio of the endpoint titer was calculated and expressed as a percentage, as follows: (titer with urea/titer without urea) × 100. IgG avidity results of ≤15% were considered low, results of 16 to 25% were considered borderline, and results of >25% were considered high.

(iii) Anti-μ-capture ELISA (Centocor, Malvern, Pa.) for the quantitation of Toxoplasma IgM was carried out according to the manufacturer’s instructions.

(iv) IgA ELISA. An ELISA (Clonatec, Paris, France) for specific IgA antibody determination was also performed according to the manufacturer’s recommendations.

Isolation in cell culture. Blood samples, cerebrospinal fluid (CSF), and urine samples from patients 1 and 4 as well as CSF from patient 2 were inoculated in human skin fibroblast cultures. No samples could be taken from patient 3.

Urine (5 to 10 ml) and CSF (0.5 ml) samples were centrifuged at 1,800 × g for 10 min. The pellets were washed twice in PBS and then inoculated into cell cultures. In the case of blood samples (0.5 ml), mononuclear cells (buffy coat) were isolated by Ficoll-Hypaque density gradient centrifugation (Lymphoprep, Nycomed, Oslo, Norway) and washed twice with PBS. The pellets were inoculated into cultures as described previously (16). Briefly, human skin fibroblasts were grown as monolayers on coverslips in shell vials. The samples were added to the shell vials, and the shell vials were incubated at 37°C in 5% CO2. The coverslips were examined for tachyzoites by Giemsa staining and by direct immunofluorescence antibody staining at 18 h to 5 days after coculturing.

Amplification and detection of toxoplasma DNA by PCR. (i) Preparation of specimens. CSF and urine samples from all patients and blood from patients 1, 3, and 4 were processed. Urine (5 to 10 ml) and CSF (0.5 ml) specimens were concentrated by centrifugation at 1,800 × g for 10 min, and the pellets were stored at −20°C until they were studied. Theuffy coat was collected from blood samples (0.5 ml) and washed twice with PBS and once in water to remove hemoglobin.

Toxoplasmosis is a disease that is endemic worldwide. It is caused by the parasitic protozoan Toxoplasma gondii. Infected immunocompetent individuals present only with mild symptoms or may even be completely asymptomatic. Nevertheless, in congenitally infected children and immunocompromised persons such as human immunodeficiency virus-infected individuals, organ transplant recipients, and cancer patients, infection causes high rates of morbidity and mortality (1, 14).

Primary T. gondii infection in pregnant women can lead to parasite transmission to the fetus via the placenta. The risk of transmission increases during gestation. Toxoplasmosis contracted during the first trimester of pregnancy induces the most serious symptoms, such as multisystemic alterations, destruction of cerebral tissue, and at worst, fetal death. In the second and third trimesters, the severity of the lesions decreases and a large percentage of infected babies are born asymptomatic. Nevertheless, the majority will later develop alterations such as mental retardation, retinochoroiditis, and organ lesions (9, 29, 31).

It has been shown that specific treatment is efficient even for patients who present with severe central nervous system lesions and that it can be used as preventive therapy in asymptomatic newborns if it is initiated in the early phase of infection and continued for an adequate period of time (24). The availability of a sensitive and reliable means of diagnosis early after infection is therefore crucial, because any delay in appropriate treatment can result in irreversible damage.

The standard toxoplasmosis diagnostic methods are based on serology. Unfortunately, their results are very difficult to interpret for immunocompromised patients, fetuses, and infants. In such cases, it is necessary to isolate the parasite in cell culture or laboratory animals or to apply molecular biology-based techniques such as PCR to confirm the diagnosis (2, 27).

Here, we present the results of a study carried out with four newborns suspected of being congenitally infected with T. gondii and whose primary diagnosis needed to be confirmed in order to begin specific treatment.

* Corresponding author. Mailing address: Servicio de Parasitología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, 28220-Majadahonda Madrid, Spain. Phone: (34-1) 5097978. Fax: (34-1) 5097966.
TABLE 1. Results of serology and *T. gondii* culture

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>ELISA IgM</th>
<th>ELISA IgA</th>
<th>IFAT IgG (IU)</th>
<th>IgG avidity (%)</th>
<th>Results of culture</th>
<th>Main clinical data</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>2,048</td>
<td>25</td>
<td>–</td>
<td>Hydrocephalus, retinal lesion, low weight</td>
</tr>
<tr>
<td>2</td>
<td>–</td>
<td>–</td>
<td>256</td>
<td>&gt;50</td>
<td>ND*</td>
<td>Cerebral calcifications, low weight</td>
</tr>
<tr>
<td>3</td>
<td>–</td>
<td>+</td>
<td>128</td>
<td>&gt;50</td>
<td>ND</td>
<td>Cerebral calcifications</td>
</tr>
<tr>
<td>4</td>
<td>–</td>
<td>–</td>
<td>1,024</td>
<td>25</td>
<td>–</td>
<td>Hydrocephalus</td>
</tr>
</tbody>
</table>

* +, positive; \( \downarrow + \), low positive; –, negative. IFAT, indirect immunofluorescence test.

\* ND, not determined.

RESULTS

The symptoms observed in the newborns suggested possible toxoplasmosis. The nonspecificity of the clinical patterns, however, did not permit a definite diagnosis. In patient 1, the serological studies revealed IgG, IgM, and IgA antibodies specific for *T. gondii*. In patients 2 and 3, only a low IgG antibody titer was observed; no other antibody classes were detected. In patient 4, the IgG titer observed was high, and a low IgM titer was also detected. IgA was not present. IgG antibody avidity studies for patients 1 and 4 revealed a ratio of 25%, generally considered to be the limiting value between the acute and chronic phases of the infection. For patients 2 and 3 the ratio was greater than 50% (Table 1).

*T. gondii* was isolated from the cell cultures in which the urine sample from patient 1 had been inoculated (Fig. 1). The first positive result could be detected in the shell vial at 18 h postinfection. All other cultured samples were negative at 7 days postinfection.

**PCR.** For patient 1, the primary PCR gave a positive result for the blood sample, which was confirmed by hybridization and nested PCR. Results of the primary PCR were negative for urine and CSF samples, but clearly positive after Southern blotting and hybridization with the internal oligoprobe and 20 h of exposure. The nested PCR also gave a positive result (Fig. 2). For patient 2, both urine and CSF samples gave positive results in the primary PCR as well as in the nested PCR. For patient 3, blood and urine samples were positive by primary and nested PCRs. However, *T. gondii* DNA could not be detected in the CSF sample. For patient 4, all samples processed were positive for *T. gondii* (Table 2).
The frequency of acquisition of *T. gondii* infection during pregnancy has been assessed to be between 1 and 14/1,000 pregnancies in different countries, and congenital *Toxoplasma* infection occurs with a prevalence of 0.2 to 2/1,000 births (29). Only a few infants show the characteristic of severe clinical damage at birth, and most congenital infections are asymptomatic. However, it has recently become clear that the long-term complications of congenital toxoplasmosis are both more frequent and more severe than was previously thought (27, 32).

The diagnosis of congenital toxoplasmosis is important for the design of patient-specific treatment. Delays in diagnosis and therapy appear to be associated with irreversible damage in some instances (18, 24). Normally, the diagnosis of congenital toxoplasmosis is based on serological demonstration of IgM, but specific IgM antibodies are frequently not present, as seen in the present study for patients 2 and 3. According to some investigators (6, 21), this phenomenon may be explained by the fact that antibody synthesis is often delayed and may not begin until some months after birth. The detection of IgA for diagnostic purposes is under evaluation, because IgA levels are higher than those of IgM in congenitally infected children. However, the specificity of IgA detected at birth must be confirmed, because maternal IgA antibodies can be detected in neonatal serum because of the sensitivity of the test (10). Therefore, for these patients in whom toxoplasma-specific IgA and IgM have been found during the neonatal period, it is necessary to carry out additional tests to confirm the results. Furthermore, IgM may be absent, as observed for patients 2, 3, and 4. Specific IgG antibody detection is not reliable because of the presence of transplacentally acquired maternal antibodies. The assay measuring the antigen-binding avidity of anti-*T. gondii* IgG antibodies separates the low-affinity antibodies produced at an early stage of infection from those with a higher binding affinity, which reflects past immunity (21, 31). Thus, the IgG avidity observed in patients 1 and 4 could indicate recent infection (not more than 6 months), which would situate the time of infection at the end of the first trimester of pregnancy. This matches the seriousness of the symptoms that were observed, which is usually associated with the transmission of parasites early in fetal life. In patients 2 and 3, the high avidity may indicate the maternal origin of these antibodies, considering the low overall IgG titer and the nonexistent immune response in the children. Consequently, the high avidity at birth reflects the avidity of maternal antibodies in the blood of the infected child. Follow-up samples are therefore needed to verify the diagnosis.

In the face of these serodiagnostic problems, the definitive diagnosis is based on the direct detection of *T. gondii*. For the present study, we chose rapid cell culture in shell vials and PCR. For patient 1, inoculation of the urine sample into the cell culture led to parasite isolation. This is the first report of the diagnosis of toxoplasmosis by using urine from immunocompetent newborns, although the isolation of *T. gondii* from the urine of immunocompromised patients has been described (25). The negative results for cultures of blood and CSF samples may originate from the small amount of available CSF, the fact that the parasitemia may have been intermittent, and the fact that whole blood or purified leukocytes may be toxic to cell culture monolayers (16).

Several studies have described the results obtained by PCR with B1 (4), P30 (8), or TGR1E (5) gene targets or a segment of the 18S rRNA gene (13). The well-characterized B1 gene was chosen for the present study because this gene appears to be conserved in all *T. gondii* isolates analyzed (26) and is present at least 35-fold in the genome (4). In the present study, we detected the *T. gondii* B1 gene in blood, urine, and CSF by gene amplification by PCR. The additional reamplification with two specific nested primers doesn’t permit an increase in the sensitivity of single PCR followed by hybridization (see data for patient 1). The high risk of contamination should be pointed out, as many protocols protecting against carryover cannot be applied to the nested PCR protocol. Consequently, it is quite important to take adequate precautions while working. Detection of *T. gondii* genes in amniotic fluid, CSF, aqueous humor, bronchoalveolar lavage, and tissue or blood samples after PCR amplification has been reported previously (3, 7, 12, 17, 19, 22, 23, 30); this is the first report of diagnosis by PCR of a urine sample. The elimination of *T. gondii* in the urine in the acute phase of disease provides an additional method of toxoplasmosis diagnosis. These samples offer the advantage of their easy accessibility by noninvasive means.

These results suggest that PCR analysis of urine samples may be a valuable approach to the diagnosis of toxoplasmosis in congenitally infected children. However, the period of time at which the parasite or its genome is present in urine is still unknown, and because only symptomatic newborns have been studied, it will be necessary to assess the utility of diagnostic PCR with urine from asymptomatic children in future studies. Furthermore, several samples should be taken for parasite detection because of its heterogeneous distribution, its elimination during the disease, and the potential presence of inhibitory factors (33).

**DISCUSSION**

**TABLE 2. PCR results**

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Blood</th>
<th>Urine</th>
<th>CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCR</td>
<td>Nested PCR</td>
<td>Hybridization</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* +, positive; -, negative; ND, not determined.

The assay measuring the antigen-binding avidity of anti-*T. gondii* IgG antibodies separates the low-affinity antibodies produced at an early stage of infection from those with a higher binding affinity, which reflects past immunity (21, 31). Thus, the IgG avidity observed in patients 1 and 4 could indicate recent and more severe than was previously thought (27, 32). The negative results for cultures of blood and CSF samples may originate from the small amount of available CSF, the fact that the parasitemia may have been intermittent, and the fact that whole blood or purified leukocytes may be toxic to cell culture monolayers (16).

Several studies have described the results obtained by PCR with B1 (4), P30 (8), or TGR1E (5) gene targets or a segment of the 18S rRNA gene (13). The well-characterized B1 gene was chosen for the present study because this gene appears to be conserved in all *T. gondii* isolates analyzed (26) and is present at least 35-fold in the genome (4). In the present study, we detected the *T. gondii* B1 gene in blood, urine, and CSF by gene amplification by PCR. The additional reamplification with two specific nested primers doesn’t permit an increase in the sensitivity of single PCR followed by hybridization (see data for patient 1). The high risk of contamination should be pointed out, as many protocols protecting against carryover cannot be applied to the nested PCR protocol. Consequently, it is quite important to take adequate precautions while working. Detection of *T. gondii* genes in amniotic fluid, CSF, aqueous humor, bronchoalveolar lavage, and tissue or blood samples after PCR amplification has been reported previously (3, 7, 12, 17, 19, 22, 23, 30); this is the first report of diagnosis by PCR of a urine sample. The elimination of *T. gondii* in the urine in the acute phase of disease provides an additional method of toxoplasmosis diagnosis. These samples offer the advantage of their easy accessibility by noninvasive means.

These results suggest that PCR analysis of urine samples may be a valuable approach to the diagnosis of toxoplasmosis in congenitally infected children. However, the period of time at which the parasite or its genome is present in urine is still unknown, and because only symptomatic newborns have been studied, it will be necessary to assess the utility of diagnostic PCR with urine from asymptomatic children in future studies. Furthermore, several samples should be taken for parasite detection because of its heterogeneous distribution, its elimination during the disease, and the potential presence of inhibitory factors (33).

**ACKNOWLEDGMENTS**

We are grateful to Beatriz Gutiérrez-Solar, Esperanza Rodríguez, and Jean Marc Lohse for advice.

This work was supported by the Fondo de Investigaciones Sanitarias (grant 96-0052-01) from the Spanish Ministry of Health.

**REFERENCES**


