

The performance of four molecular methods for the laboratory diagnosis of congenital toxoplasmosis in amniotic fluid samples

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ABSTRACT

Introduction: Toxoplasmosis may be life-threatening in fetuses and in immune-deficient patients. Conventional laboratory diagnosis of toxoplasmosis is based on the presence of IgM and IgG anti-*Toxoplasma gondii* antibodies; however, molecular techniques have emerged as alternative tools due to their increased sensitivity. The aim of this study was to compare the performance of 4 PCR-based methods for the laboratory diagnosis of toxoplasmosis. One hundred pregnant women who seroconverted during pregnancy were included in the study. The definition of cases was based on a 12-month follow-up of the infants. **Methods:** Amniotic fluid samples were submitted to DNA extraction and amplification by the following 4 *Toxoplasma* techniques performed with parasite B1 gene primers: conventional PCR, nested-PCR, multiplex-nested-PCR, and real-time PCR. Seven parameters were analyzed, sensitivity (Se), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (PLR), negative likelihood ratio (NLR) and efficiency (Ef). **Results:** Fifty-nine of the 100 infants had toxoplasmosis; 42 (71.2%) had IgM antibodies at birth but were asymptomatic, and the remaining 17 cases had non-detectable IgM antibodies but high IgG antibody titers that were associated with retinochoroiditis in 8 (13.5%) cases, abnormal cranial ultrasound in 5 (8.5%) cases, and signs/symptoms suggestive of infection in 4 (6.8%) cases. The conventional PCR assay detected 50 cases (9 false-negatives), nested-PCR detected 58 cases (1 false-negative and 4 false-positives), multiplex-nested-PCR detected 57 cases (2 false-negatives), and real-time-PCR detected 58 cases (1 false-negative). **Conclusions:** The real-time PCR assay was the best-performing technique based on the parameters of Se (98.3%), Sp (100%), PPV (100%), NPV (97.6%), PLR (∞), NLR (0.017), and Ef (99%).

Keywords: Congenital toxoplasmosis. Congenital infection. Molecular diagnosis. PCR. Quantitative PCR. Real-time PCR.

INTRODUCTION

Toxoplasmosis is a worldwide endemic protozoan disease that is acquired mainly through infected meat. It is considered a major health problem in pregnant women and in immune-compromised patients. Primary maternal *Toxoplasma* infection may be health-threatening for the fetus, or may even cause death in utero, depending on the date of transmission, parasite burden, parasite genotype, and host susceptibility. The reactivation of a latent infection under immune-deficiency conditions, such as acquired immunodeficiency syndrome (AIDS) and organ transplantation, can cause fatal toxoplasmic encephalitis.

Toxoplasmosis is also an important cause of chorioretinitis, especially in individuals with an impaired immune system. In these conditions, a rapid and accurate diagnosis is required to enable initiation of anti-parasitic treatment as early as possible to improve the prognosis¹.

While conventional laboratory diagnosis is based on serology parameters, such as the presence of IgM and IgG anti-*Toxoplasma* antibodies and, to a minor extent, on the parasite isolations in mice or in cell culture that are performed only in reference centers, molecular techniques, such as polymerase chain reaction (PCR), have recently emerged as alternative tools to improve the laboratory diagnosis of toxoplasmosis due to their increased sensitivity²⁻⁴. Therefore, the diagnosis of clinically relevant congenital toxoplasmosis now relies on molecular techniques, although these techniques are prone to performance variation due to a large diversity of target genes and primers⁵, in addition to factors associated with the proficiency of laboratories in the optimization of PCR conditions².

Recently, the superiority of quantitative amplification methods over older techniques, such as one-round and nested-PCR, has been reported²⁻⁴, but there is a lack of information regarding the performance of such methods in Brazil.

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In an attempt to take the first step toward a consensus on the best molecular technique for diagnosing congenital toxoplasmosis in Brazilian patients, we compared 4 PCR-based methods performed with parasite B1 gene primers, varying from conventional systems containing 1 or 2 rounds of amplification to real-time PCR, to determine the best-performing technique. The 4 amplification methods were based on B1 gene primers because in previous experiments analyzing a significant number of amniotic fluid samples of Brazilian women, B1 gene primers performed better than rep529 and ribosomal deoxyribonucleic acid (rDNA) primers⁵.

Study design

One hundred pregnant women receiving prenatal care at public hospitals of the State of São Paulo and presenting with toxoplasmosis seroconversion, i.e., the presence of positive immunoglobulin M (IgM) and significant titers of immunoglobulin G (IgG) anti-*Toxoplasma gondii* antibodies, were screened by commercial immune enzyme assays, chemiluminescence, or immunofluorimetry, depending on local availability. This initial serology was confirmed by indirect immunofluorescence or IgM capture according to local laboratory availability and expertise. The IgG avidity test was used in 16 of the 100 patients. Exclusion criteria were inadequate amniotic fluid samples, due to mixture with either blood or meconium, or sample insufficiency. Among the 100 pregnant women, 23 underwent amniocentesis during the 16th week of gestation, 20 during the 17th week, 18 during the 18th week, 13 during the 19th week, 5 during the 20th or 21st weeks, 6 during the 22nd week, 5 during the 23rd week, 3 during the 24th week, and 2 during the 25th week of pregnancy. The procedure was performed after the women signed informed consent forms (Institutional Ethics Committee protocol number 0715/2011). All 100 women received treatment with 3g/day of spiramycin, which was prescribed as soon as the attending physician received the serology results and continued until 1 week before the scheduled date of delivery to minimize the risk of bleeding. Patients were informed of the risks of amniocentesis, as well as the nucleic acid procedures that would be performed in the context of the research, and the participants consented to all subsequent testing.

Case definition

A case of congenital toxoplasmosis was confirmed when the newborn/infant had anti-*T. gondii* IgM antibodies and/or high IgG antibody titers (significantly higher than the maternal titers at birth and >300IU/ml in immune enzyme assays at any time point) at birth or at some point during the 12-month follow-up. In the absence of positive IgM antibodies, high IgG antibody titers should be associated with signs and symptoms suggestive of toxoplasmosis and/or cranial ultrasound and/or eye fundus abnormalities. These complementary tests, together with serology, were repeated every 3 months during the follow-up.

METHODS

DNA extraction and assessment of DNA concentration

Deoxyribonucleic acid (DNA) extraction from amniotic fluid samples was performed with a commercial kit (QIAamp DNA

mini kit, QIAGEN Inc., Valencia, California, USA) according to the manufacturer's instructions. DNA concentration was estimated by ultraviolet (UV) spectrophotometry at 260nm (Nanodrop 1000, Thermo Scientific, USA).

Amplifications

Prior to the specific *Toxoplasma gondii* amplifications, all DNA samples yielded positive results with human beta-actin primers⁶, confirming that there were no PCR inhibitors present. *Toxoplasma gondii* amplifications were performed in a total volume of 25µl, consisting of 100ng of amniotic fluid sample DNA, 200µM of dNTP, 0.2µM of primers, 2.0mM of MgCl₂, and 1.25units of Taq DNA polymerase (GE Healthcare, USA), following stringent procedures to minimize the risk of carryover⁷. A total of 40 cycles/round were performed, with annealing temperatures of 55°C for the outer primer pair JW62/JW63 and 62°C for the inner primer pair B22/B23, generating amplification products of 286 and 115bp, respectively⁵. The DNA-PCR was performed with primers JW62/JW63; the nested-PCR was performed with primers JW62/JW63 and B22/B23; and the multiplex-nested-PCR was performed with the same JW62/JW63 and B22/B23 primers used for *T. gondii* detection and other three primer pairs for the simultaneous detection of cytomegalovirus (CMV), herpes simplex virus (HSV), and Epstein-Barr virus (EBV)^{8,9}. The composition of the parasite B1 gene primers was: JW63-GCACCTTTCGGACCTCAACAACCG; JW62-TTCTCGCCTCATTCTGGGTCTA C; B22-AACGGGCGAGTAGCACCTGAGGAGA; and B23-TGGGTCTACGTTCGATGG ATGACAACT. The fourth amplification procedure was a 40-cycle real-time PCR with primers B22/B23. The concentrations of primers and template DNA were the same as those used for the conventional amplifications, and the master mix was prepared with a commercial kit (QuantiFast SYBR Green PCR, QIAGEN). Cycles were performed at 95°C for 15 seconds at an annealing temperature of 62°C for 50sec, ending with temperatures between 82 and 83°C on the melting curve. The slope was -3.54; the efficiency was in the range of 92-100%; and the detection limit was 1 parasite in 70% and 10 parasites in 100% of experiments (Thermocycler Step One Plus, Applied Biosystems, Foster City, California, USA). Samples were tested in triplicate with real-time PCR, but results were considered only in a binary manner (positive or negative).

RESULTS

All 100 women in the study had positive IgM and IgG anti-*T. gondii* antibodies as assessed by screening serology and also by a confirmatory test. Unfortunately, the IgG avidity test results could not be used to aid the laboratory diagnosis because these data were only available for 16 of the 100 patients, and the percent avidity was inconclusive in 10 of these 16 cases (data not shown).

The assessment of newborns by clinical, serological, and complementary exams and the monitoring of infants until the age of 12 months revealed that among the 100 total cases, 59 had confirmed toxoplasmosis; 42 (71.2%) newborns had IgM

antibodies at birth but were asymptomatic, and the remaining 17 (28.8%) newborns had high IgG titers (> 300IU/ml) in the serology tests. These high titers were associated with chorioretinitis in 8 (13.5%) infants; abnormal cranial ultrasound in 5 (8.5%) infants; and signs/symptoms suggestive of infection, such as marked jaundice, hepatomegaly, and splenomegaly that regressed after treatment, in 4 (6.8%) infants. Among the 59 confirmed cases, 50 were positive based on conventional PCR (9 false-negative results and no false-positive results), 58 based on nested-PCR (1 false-negative and 4 false-positive results),

57 based on multiplex-nested-PCR (2 false-negative results and no false-positive results), and 58 based on real-time PCR (1 false-negative and no false-positive results).

Parameters of sensitivity (Se), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (PLR), negative likelihood ratio (NLR) and efficiency (Ef) of the 4 amplification techniques were analyzed based on the confirmation of positive and negative cases according to their clinical and laboratory evolution until the age of 12 months. The 7 analyzed parameters are displayed in **Table 1**.

TABLE 1 - The performance of 4 PCR-based techniques for the molecular diagnosis of congenital toxoplasmosis.

Technique	Se (95% CI)	Sp (95% CI)	PPV (95% CI)	NPV (95% CI)	PLR	NLR	Ef (%)
Conventional PCR	84.7 (0.73 – 0.93)	100.0 (0.91 – 1.00)	100.0 (0.94 -1.00)	82.0 (0.69 – 0.91)	§	0.15	91.0
Nested PCR	98.3 (0.91 – 0.99)	90.2 (0.77 – 0.97)	93.5 (0.84 – 0.98)	97.4 (0.86 – 0.99)	10.07	0.018	95.0
Multiplex-nested-PCR	96.6 (0.88 – 0.99)	100.0 (0.91 – 1.00)	100.0 (0.94 – 1.00)	95.3 (0.84 -0.99)	§	0.034	98.0
Real-time PCR	98.3 (0.91 – 0.99)	100.0 (0.91 – 1.00)	100.0 (0.94 – 1.00)	97.6 (0.87 – 0.99)	§	0.017	99.0

Se: sensitivity; Sp: specificity; PPV: positive predictive value; NPV: negative predictive value; PLR: positive likelihood ratio; NLR: negative likelihood ratio; Ef: efficiency; PCR: polymerase chain reaction; §: infinite; CI: confidence interval.

DISCUSSION

The effectiveness of different target genes and primers in the amplification of *T. gondii* DNA in different types of biological samples has long been evaluated in the literature; however, there is a lack of consensus on this topic, at least in non-European countries, and performance is strongly dependent on the proficiency of laboratories in the optimization of the PCR^{2,5}.

Reischl et al.¹⁰, in a European study, used real-time PCR (LightCycler) with rep529 and B1 gene primers to evaluate 51 *Toxoplasma gondii*-positive amniotic fluid samples and found a 10-100-fold higher sensitivity for the PCR assay that targeted the 529-bp repeat element of *Toxoplasma gondii*.

Edvinsson et al.¹¹, also in Europe, investigated whether real-time PCR could be used to monitor parasitemia in the blood of a transplant recipient presenting with toxoplasmosis. The results demonstrated that detection of the 529-bp repeat element increased the diagnostic sensitivity and accuracy compared with the assessment of the B1 gene only. These authors also added an internal amplification control that did not apparently affect the

PCR performance and was useful for monitoring PCR inhibition by non-specific DNA in the LightCycler instrument.

One of the first Brazilian studies on the molecular diagnosis of congenital toxoplasmosis was conducted by Vidigal et al.¹² in 2005. The authors analyzed 86 amniotic fluid samples from women who seroconverted during pregnancy in the State of Minas Gerais. Samples were inoculated in mice and submitted to *T. gondii* DNA amplification by a nested-PCR. Newborns were also evaluated by *T. gondii* serology, skull x-ray, transfontanel ultrasound, fundoscopic examination, and lumbar puncture. PCR was positive in 7 cases and negative in 79 (7/86 or 8.1%) cases. Among the PCR-positive cases, two were negative after mouse inoculation; among the PCR-negative cases, 3 had clinical evidence of toxoplasmosis and 1 was positive after mouse inoculation. PCR showed a sensitivity of 62.5% and specificity of 97.4% whereas mouse inoculation had a sensitivity of 42.9% and a specificity of 100%. At that time, the authors considered PCR to be a promising diagnostic method.

In 2009, Okay et al.⁵ conducted a study in the State of São Paulo, Brazil. They analyzed 467 amniotic fluid samples from women who had seroconverted during pregnancy and found 189 (40.5%) positive samples by one-round amplifications:

120 (63.5%) with the *T. gondii* B1 gene, 24 (12.7%) with the parasite AF146527 sequence (also called rep529), 45 (23.8%) with both, rep529 and the B1 gene, and none with rDNA (ribosomal DNA). In the same study, 50 previously negative one-round PCR samples were chosen by computer-assisted randomization analysis and were re-tested by a nested-PCR based on the parasite B1 gene, which detected 9 additional cases (9/50 or 18%). The authors concluded that there was significant performance variation among one-round and nested-amplification systems and showed that the most sensitive method of those tested was a nested-PCR assay with the outer primers JW62/JW63 and the inner primers B22/B23 from the parasite B1 gene compared with other target genes, such as rep529, and rDNA. However, in those experiments, the rep529 primers and amplification products were not suitable for real-time PCR.

In contrast, some studies reported an increased sensitivity of PCR targeting the rep529 sequence over that of the B1 gene²⁻⁴; these findings were corroborated by Menotti et al.¹³, who developed a new 5'-nuclease real-time PCR assay targeting the rep529 sequence (TaqMan-rep529-PCR) to diagnose toxoplasmosis and assess treatment follow-up. Initially, these authors analyzed 144 clinical specimens for the presence of *T. gondii* DNA by a polymerase chain reaction-enzyme-linked immunosorbent assay (PCR-ELISA) assay targeting the parasite B1 gene (B1-PCR-ELISA) and found 15 positive samples, all of which were from patients with clinically proven toxoplasmosis that were negative by the B1-PCR-ELISA and positive according to the TaqMan-rep529-PCR. Then, the same authors analyzed 203 consecutive clinical specimens during a 4-month period. The diagnosis of toxoplasmosis in two patients was made only by the TaqMan-rep529-PCR and not by the B1-PCR-ELISA. Additionally, samples from a patient with cerebral and disseminated toxoplasmosis, who had already been tested using a B1 real-time PCR assay, were evaluated by the TaqMan-rep529-PCR in a Light Cycler real-time PCR assay targeting the same repetitive rep529 sequence. The TaqMan-rep529-PCR assay was able to detect positive samples and had a mean gain of 7.1 following 3.3 amplification cycles compared with the B1 real-time PCR assay. These authors concluded that the 5'-nuclease real-time PCR assay developed for the rep529 sequence was more sensitive than those using the B1 gene.

However, Wahab et al.¹⁴ showed that the rep529 element was absent in 4.8% of human *T. gondii*-positive samples, suggesting that the B1 gene should be considered a preferred diagnostic target. Nevertheless, the rep529 sequence has been the most used sequence in European studies, including a study that analyzed the performance of 3 molecular methods for the amplification of *T. gondii* DNA in amniotic fluid samples, namely a commercial kit (nested-PCR) and two in-house methods (a conventional PCR and a real-time PCR). This evaluation was based on a *T. gondii* DNA serial dilution, 3 amniotic fluid samples inoculated with *T. gondii* at different concentrations, and 33 amniotic fluid samples from a clinical cohort. The *T. gondii* DNA serial dilution assay showed a much lower sensitivity for the commercial kit than for the two in-house methods. Moreover, of the 12 proven congenital toxoplasmosis cases among the cohort, 91.7% were detected by the in-house assays, whereas only 50%

were detected by the commercial kit¹⁵. This lack of sensitivity was attributed to the presence of PCR inhibitors.

In a recent Brazilian study¹⁶, a real-time PCR (qPCR) targeting the parasite rep529 sequence was positive in 72 (48%) blood samples from 150 infected newborns. Among the infants with active retinochoroiditis, 68% had positive qPCR results, while positivity was much lower (29%, $p=0.009$) in the absence of ocular involvement. The authors concluded that the qPCR acted as a prognostic factor and that the positivity of samples was associated with the presence of retinochoroidal lesions, with an odds ratio of 2.8. In that study, it is clear that the qPCR assay was used to evaluate the prognosis of newborns with respect to eye injuries and was not used for the purpose of diagnosis, given that the biological material used was blood. This methodology explains the low (48%) percentage of newborns with confirmed congenital toxoplasmosis and positive qPCR results, contrasting with the 58/59 confirmed cases (98.3%) detected in the amniotic fluid samples of the present study. Nevertheless, as the target gene (rep529 sequence versus B1 gene) and the biological material (peripheral blood versus amniotic fluid samples) were different, prospective studies comparing real time PCR performed with rep529 primers or B1 gene primers in the same type of biological samples are needed. It is also important to clarify that the rep529 pair of primers tested in our laboratory in the experiments published in 2009⁵ are not suitable for real-time PCR, contrasting with the rep529 primers described in that recently published Brazilian report¹³.

In the present study, 100 women who seroconverted during pregnancy were included in the study. Among the 100 suspected cases, 59 were confirmed and 41 were excluded after a 12-month follow-up during which the infants were clinically re-evaluated and also analyzed by means of serology, as well as other exams, e.g., ocular funduscopy and cranial ultrasound or computerized tomography in older infants, repeated every 3 months. Amniotic fluid samples were subjected to 4 different molecular tests performed with the parasite B1 gene primers, always by the same technician in the same laboratory facility. Importantly, neither technique was able to detect all 59 confirmed cases in this study. However, it is important to mention that we did not stratify the cases according to gestational age at the time of transmission, and this parameter seems to be a determinant of the positivity found by different molecular methods in the congenital toxoplasmosis model. For example, Sterkers et al.¹⁷ analyzed the performance of an optimized PCR assay tested on amniotic fluid samples, placenta, and cord blood in a cohort of 344 pregnant women who underwent amniocentesis and whose children were followed for 1 year. The authors analyzed the PCR results according to the gestational age at the time of maternal infection and inferred accurate post-test predictive values for diagnosis. They concluded that despite the persistence of false-negatives, using a highly accurate molecular diagnostic test and the post-test risk curves, it is possible to predict the actual risk of congenital toxoplasmosis at different gestational ages.

According to the analyzed parameters for the 4 molecular techniques and the TDR (UNICEF, UNDP, World Bank, WHO) recommendations developed to guide laboratory diagnostic procedures, including Nucleic Acid Tests (NATs)¹⁸, DNA-PCR

should not be used due to its relatively low (84.7%) sensitivity. This technique failed to detect 9 positive cases, but it did not yield any false-positive results. The nested-PCR technique had a specificity of 90.2% and thus did not meet the specificity criterion for laboratory diagnostic procedures (specificity >95%). It also produced 4 false-positive results, possibly caused by carry-over, which is a known drawback of this type of amplification, even when performed by experienced individuals⁷. Among the methods that complied with the recommended sensitivity and specificity levels, i.e., the multiplex-nested-PCR and real-time PCR techniques, the multiplex-nested-PCR did not produce any false-positive results but detected 1 fewer case than the nested-PCR technique, even though both techniques were performed with the same *Toxoplasma* primer pairs. This difference was most likely due to competition for the use of reagents, as there were 4 primer pairs in each multiplex-nested-PCR to allow for simultaneous detection of *T. gondii*, CMV, HSV, and EBV^{8,9}. Among the 4 proposed methods, the real-time PCR technique detected 58 of the 59 possible cases did not produce any false-positive results and was the best-performing test considering all 7 analyzed parameters, a sensitivity of 98.3%, specificity of 100%, PPV of 100%, NPV of 97.6%, an infinite PLR, a NLR of 0.017, and an efficiency of 99%, corroborating the findings of previous studies²⁻⁴ that highlighted the advantages of real-time PCR. Moreover, this technique is able to determine the number of parasites/target gene copy number, enabling follow-up of treated patients, and has a short run time, reducing the risk of carry-over and handling of DNA intercalating substances.

In conclusion, in this study performed on 100 amniotic fluid samples from pregnant women living in the State of São Paulo, the best-performing technique, based on the parameters of sensitivity, specificity, positive and negative predictive values, positive and negative likelihood ratios, and efficiency, was the real-time PCR technique with primers B22/23. In the future, we intend to analyze these same amniotic fluid samples with primers of the parasite rep529 sequence using real-time PCR to compare our results with those of other studies.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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