Evaluation of serological and molecular tests used to identify
*Toxoplasma gondii* infection in pregnant women attended in a public
health service in São Paulo state, Brazil

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**ABSTRACT**

Toxoplasmosis during pregnancy can have severe consequences. The use of sensitive and specific serological and molecular methods is extremely important for the correct diagnosis of the disease. We compared the ELISA and ELFA serological methods, conventional PCR (cPCR), Nested PCR and quantitative PCR (qPCR) in the diagnosis of *Toxoplasma gondii* infection in pregnant women without clinical suspicion of toxoplasmosis (G1 = 94) and with clinical suspicion of toxoplasmosis (G2 = 53). The results were compared using the Kappa index, and the sensitivity, specificity, positive predictive value and negative predictive value were calculated. The results of the serological methods showed concordance between the ELISA and ELFA methods even though ELFA identified more positive cases than ELISA. Molecular methods were discrepant with cPCR using B22/23 primers having greater sensitivity and lower specificity compared to the other molecular methods.

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**1. Introduction**

Toxoplasmosis, an infection caused by the obligate intracellular parasite *Toxoplasma gondii*, affects mammals and birds worldwide (Dubey, 2008; Robert-Gangneux and Dardé, 2012). In humans, the disease can be severe especially during pregnancy, as the parasite can cross the placental barrier and infect the fetus with serious and even fatal consequences (Robert-Gangneux and Dardé, 2012).

The prevalence of gestational toxoplasmosis is high in many regions of Brazil (Câmara et al., 2015; Gontijo et al., 2015; Lopes-Mori et al., 2013; Moura et al., 2013; Porto et al., 2008; Reboças et al., 2011; Spalding et al., 2005; Sroka et al., 2010) including the northwestern region of São Paulo State (64.4% – Mattos et al., 2011a) (Fig. 1). Early diagnosis and anti-parasite treatment can reduce the severity of the fetal disease, but complications, such as microcephaly, hydrocephalus, cerebral calcifications, retinocoroidites, and mental retardation, can occur if cases remain untreated (Bittencourt et al., 2012; Fochi et al., 2015; McLeod et al., 2012, 2014; Rodrigues et al., 2009; Sroka et al., 2010).

The diagnosis of toxoplasmosis is challenging because the clinical manifestations are often nonspecific. Thus, the use of sensitive serological and molecular tests is extremely important to identify the disease early (Bichara et al., 2012; Lago et al., 2014; McLeod, 2014; Robert-Gangneux and Dardé, 2012). The aim of this study was to compare the serological and molecular methods used to diagnose...
toxoplasmosis in pregnant women treated at a teaching hospital in the northwestern region of São Paulo State.

2. Material and methods

2.1. Ethics statement

This study was approved by the Ethics Committee of the Medicine School in São José do Rio Preto (FAMERP-CAAE 32259714.8.0000.5415).

2.2. Patients and clinical samples

This is a retrospective study that evaluated pregnant women treated at the High-risk Antenatal Care and Fetal Medicine Outpatient Clinic of the Fundação Faculdade Regional de Medicina, Hospital de Base (FUNFARME), São José do Rio Preto, São Paulo State, Brazil. The pregnant women were characterized in 2 groups: G1 – Pregnant women without clinical suspicion of toxoplasmosis, who had been referred to the clinic due to other complications (n = 94) and G2 – high-risk pregnant women with suspicion of toxoplasmosis and/or positive for IgM anti-T. gondii antibodies at some time during pregnancy (n = 53).

Peripheral blood was collected from all subjects in a dry tube for serological analysis and in a tube with ethylenediaminetetraacetic acid (EDTA) for DNA extraction and molecular tests. Serological and molecular analyses were performed in the Immunogenetics Laboratory, Molecular Biology Department, FAMERP, São José do Rio Preto, São Paulo, Brazil. Of the 53 pregnant women in G2, 50 were also submitted to amniocentesis to investigate T. gondii in amniotic fluid. Amniotic fluid was sent to the reference laboratory of the São Paulo State Health Department (Dr. Vera Pereira-Chioccola) for conventional polymerase chain reaction (cPCR). All pregnant women are routinely screened in the High-risk Antenatal Care and Fetal Medicine Outpatient Clinic for TORSCH (Toxoplasmosis, Rubella, Syphilis, Cytomegalovirus, Hepatitis and HIV) (Gonçalves et al., 2010).

2.3. Serological diagnosis

The presence of anti-T. gondii was confirmed by enzyme linked immunosorbent assay (ELISA, DiaSorin, Italy) using the ETI-TOXOK-A reverse plus kit for IgA, ETI-TOXOK-M reverse plus plus kit for IgM and ETI-TOXOK-G plus kit for IgG, and enzyme linked fluorescent assay (ELFA, Biomerieux, France) using the Vidas®Toxo IgM kit for IgM, Vidas®Toxo IgG II kit for IgG and Vidas®Toxo IgG avidity kit for IgG avidity. The detection of IgA and IgM antibodies was performed by capture ELISA. All samples that were positive for IgG and IgM anti-T. gondii antibodies by ELFA were also assayed using the ELFA IgG avidity test. Low-avidity antibodies (<25%) are indicative of recent infection. ELFA was performed in automated equipment (Mini Vidas, Biomerieux, France). Both tests were performed according to manufacturer’s instructions. Samples were considered positive for IgG antibodies by ELISA when the concentration was >15 IU/mL and negative when the IgG concentration was ≤15 IU/mL. Results were considered positive for IgA antibodies by ELISA when the IgA concentration was >5 AU/mL, and negative when the concentration was ≤5 AU/mL. For the IgM ELISA test, the absorbance values of the samples were compared with the average cut-off point; samples were considered positive when the absorbance values were higher than or equal to the cut-off point with the remaining samples being considered negative. By ELFA, samples were considered positive for IgG antibodies when the value was ≥8 IU/mL, indeterminate from ≥4 to <8 IU/mL and negative when <4 IU/mL. For IgM antibodies, ELFA results were positive when the reagent index was ≥0.65 IU/mL, indeterminate from <0.65 to ≥0.55 IU/mL and negative <0.55 IU/mL. Results demonstrated low IgG avidity when the IgG antibody concentration was <0.200, intermediate avidity was between 0.200 and 0.300 and high avidity when the IgG antibody concentration was ≥0.300.

2.4. Molecular diagnosis

2.4.1. Genomic DNA extraction

The genomic DNA was extracted from 5 mL of peripheral blood collected in EDTA using a commercial kit (Qiamp DNA blood mini kit,
Qiagen, Germany) according to the protocol described by Mattos et al. (2011b). The extracted DNA was stored in a freezer at −20 °C until polymerase chain reaction (PCR).

2.4.2. Identification of the Toxoplasma gondii B1 gene

2.4.2.1. Conventional polymerase chain reaction (cPCR). cPCR was performed to identify T. gondii DNA in blood samples. Two cPCR reactions were performed, one with the JW62/63 primer pair and the other with the B22/23 primer pair. The B22 (sense: 5′-AACGCGGAGTAGCCTAGCTGAGGAGA-3′) and B23 primers (anti-sense: 5′-TGGGTCTACGTCGATGGCATGACAACT-3′) amplify a 115-base-pair sequence of a specific repetitive region of the B1 gene (accession numbers: B1 gene T. gondii = GenBank: KR559682.1) (Burg et al., 1989; Colombo et al., 2005). The PCR mixture was prepared using 8.5 μL of nucleic acid-free water (Promega, USA); 12.5 μL of GoTaq Green Master Mix (Promega, USA) and 1.0 μL of each of the JW62 and B23 primers (25 pmol each – IDT, USA). DNA from patients and controls (5 μL in [100 ng/μL]) were added to each of the PCR mixture in a final volume of 25 μL. The PCR cycling conditions consisted of an initial denaturation step at 95 °C for 5 min, 35 amplification cycles of 45 s at 95 °C, 45 s at 62 °C and 45 s at 72 °C with a final extension of 5 min at 72 °C in a thermocycler (Verity, Applied Biosystems, USA). The PCR products were electrophoresed in 1.5% agarose gel using SYBR Safe stain (Invitrogen, USA).

2.4.2.2. Nested PCR. cPCR was performed using the JW62 (antisense: 5′-TCTCCGCTTATTGCTGCTAC-3′) and JW63 primer pair (Sense: 5′-GCACCTTTGGACCTCAAACC-3′), which amplifies a fragment of 286 base pairs of the T. gondii B1 gene. The PCR mixture was prepared using 6.5 μL nucleic acid-free water (Promega, USA), 12.5 μL of GoTaq Green Master Mix (Promega, USA) and 0.5 μL of each of the JW62 and JW63 primers (10 μM each primer – IDT, USA). DNA from patients and controls (5 μL in [100 ng/μL]) were added to the PCR mixture in a final volume of 25 μL. The PCR cycling conditions consisted of an initial denaturation step at 95 °C for 5 min, 40 amplification cycles of 45 s at 95 °C, 45 s at 55 °C and 45 s at 72 °C with a final extension of 5 min at 72 °C in a thermocycler (Verity, Applied Biosystems, USA). The PCR products were electrophoresed in 1.5% agarose gel using SYBR Safe stain (Invitrogen, USA).

The amplified product was subjected to a second PCR (Nested PCR) using the B22/23 primer pair following the protocol published by Okay et al. (2009) with modifications. The PCR mixture was prepared for the second reaction using 6.5 μL nucleic acid-free water (Promega, USA), 12.5 μL of GoTaq Green Master Mix (Promega, USA) and 0.5 μL of each of the JW62 and JW63 primers (10 μM each primer – IDT, USA). Five microliters from the first amplification reaction using the JW62/63 primer pair were added. The PCR cycling conditions consisted of an initial denaturation step at 95 °C for 5 min, 25 amplification cycles of 45 s at 95 °C, 45 s at 55 °C and 45 s at 72 °C with a final extension of 5 min at 72 °C in a thermocycler (Verity, Applied Biosystems, USA). The PCR products were electrophoresed in 1.5% agarose gel using SYBR Safe stain (Invitrogen, USA).

Statistical analyses used the IBM SPSS software v.23 to determine the Kappa index (κ) and GraphPad Stat Software v. 3.06 was used to determine the sensitivity, specificity, positive predictive value and negative value. Sensitivities and specificities were calculated as:

\[
\begin{align*}
\text{Sensitivity} & = \frac{TP}{TP + FN} \\
\text{Specificity} & = \frac{TN}{TN + FP}
\end{align*}
\]

with a final extension of 30 s at 50 °C. The primers and probe used in this analysis have been described by Gunel et al. (2012).

Ultrapure water and DNA extracted from T. gondii (RH strain) were included as negative and positive controls, respectively in all PCR reactions (cPCR, Nested PCR and qPCR). To control the course of DNA extraction and check for PCR inhibitors, all samples were assayed using the HGH primer (Accession number: HGH = GenBank: U55206.1 – sense: 5′- GCTTCCACAGATCCTGAGTACACACTG-3′ and antisense: 5′-TCAGGATTCTG TTGTGTTCC-3′), which amplifies a 400-base-pair fragment of the human growth hormone gene.

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\]
reported by other authors (Bobić et al., 2012; Câmara et al., 2015; Murata et al., 2016). No statistically signiﬁcant difference was found between the serological methods used to identify T. gondii infection in neonates, 2 of which were automatic (MEIA and ELFA IgM), reported sensitivity and speciﬁcity of 60.9% and 100.0%, respectively against IgM indirect immunoassay (IFAT – 59.6% and 91.7%, respectively) and ELISA IgA (57.1% and 100.0%, respectively). However, a study by Maudry et al. (2009) that used 6 automated methods to test samples from pregnant women and immunocompromised patients found sensitivity ranging from 89.7% to 99.4% and speciﬁcity between 99.1% and 100%.

The better sensitivity displayed by automated methods can improve the diagnosis of T. gondii infection in particular during pregnancy, where early diagnosis and treatment of pregnant women have signiﬁcantly reduced mother-to-child transmission, thereby reducing the risk of serious sequelae in the fetus (Pomares and Montoya, 2016).

The low speciﬁcity of IgG antibodies is mainly related to high rates of seroprevalence in the region covered by this study and the life-long persistence of these antibodies in the host, even without the disease, as demonstrated in studies of individuals without clinical symptoms (Fromont et al., 2009; Obaidat et al., 2015; Rodrigues et al., 2015).

The IgG antibody test is not commonly used and its use in the diagnosis of gestational infection has been questioned, even though this test assists in the diagnosis of congenital infection (Faure et al., 1999; Jenum and Stray-Pedersen, 1998; Li et al., 2016; Montoya, 2002; Murat et al., 2013a; Pinon et al., 2001). In this study, IgA positivity in the group of pregnant women and immunocompromised patients found sensitivity ranging from 89.7% to 99.4% and speciﬁcity between 99.1% and 100%.

The sensitivity, speciﬁcity, and positive and negative predictive values were calculated for each serological and molecular test separately. The results are shown in Table 3. Table 4 shows the comparison between the avidity with serological and molecular test in clinical samples.

### 4. Discussion

This study compared serological and molecular methods used to identify T. gondii infection in pregnant women with and without clinical suspicion of gestational toxoplasmosis in the northwestern region of São Paulo State. The rate of infection of pregnant women, as detected by serological methods, was high in this study, which is in line with other studies conducted in Brazil (Lopes-Mori et al., 2013; Sroka et al., 2010; Vaz et al., 2010) and other countries (Harma et al., 2004; Marquez and Etcheverry, 2009; Pappas et al., 2009; Sanchez-Gutierrez et al., 2003). Serological methods (ELISA and ELFA), widely used to identify T. gondii infection in Brazil, are highly sensitive and specific (Avelino et al., 2014; Bichara et al., 2012; Câmara et al., 2015; Murata et al., 2016). No statistically signiﬁcant difference was found between the serological methods in this study. However, 4 samples were discrepant for IgM between the ELFA and ELISA methods, with ELFA having the highest number of positive cases. These samples showed high avidity, which is indicative of chronic infection; 2 were also positive for IgA. The presence of these antibodies, especially IgM in chronic infections, has also been reported by other authors (Bobić et al., 1991; Liesenfeld et al., 1997; Spalding et al., 2003). IgM antibodies are widely used to identify the disease during acute infections, but they remain detectable for long periods, and thus further tests, such as the IgG avidity test, IgE assay and parasitological tests need to be performed to conﬁrm acute infections (Dhakal et al., 2015).

In this study, ELFA detected more cases positive for IgM antibodies in G2 and more cases with IgG antibodies in G1. This higher positivity may be related to the fact that ELFA is an automated method; for some authors, automated methods are more sensitive and speciﬁc (Calderaro et al., 2008; Kasper et al., 2009; Maudry et al., 2009; Murat et al., 2013b; Petersen et al., 2005; Prusa et al., 2010; Rodrigues et al., 2005; Wilson et al., 1997). In this study, ELFA and ELISA had sensitivities of 100% for IgG, and 79.2% and 75.5% for IgM, respectively (Table 3). A study conducted by Rodrigues et al. (2009) using 4 serological methods to identify anti-T. gondii infection in neonates, 2 of which were automated (MEIA and ELFA IgM), reported sensitivity and speciﬁcity of 60.9% and 100.0%, respectively against IgM indirect immunofluorescent antibody test (IFAT – 59.6% and 91.7%, respectively) and ELISA IgA (57.1% and 100.0%, respectively). However, a study by Maudry et al. (2009) that used 6 automated methods to test samples from pregnant women and immunocompromised patients found sensitivity ranging from 89.7% to 99.4% and speciﬁcity between 99.1% and 100%.

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patients with clinical suspicion of toxoplasmosis was high (56.6%); thus, its use may improve the diagnosis of T. gondii infection especially when used with IgM and IgG antibodies (Li et al., 2016; Murata et al., 2016; Pomares and Montoya, 2016; Villard et al., 2016).

Detection of T. gondii DNA by molecular methods is quite controversial as there are several techniques and markers to identify the DNA of the parasite, and there is no consensus on which protocol is the best. Moreover, the treatment of pregnant women can modify the results of serological and molecular tests, as has been described by some authors (Lago et al., 2014; Lefevere-Pettazzoni et al., 2007; McLeod, 2014; Okay et al., 2009; Robert-Gangeux and Dardé, 2012).

In this study, there were no statistically significant differences between the molecular methods investigated. In G2, 4 samples (7.5%) were positive by cPCR using the B22/23 primers. There was no detectable DNA of T. gondii using the JW62/63 primer pair; however, after being subjected to a second PCR (nested PCR) 3 (5.7%) were positive, 2 of which were also positive by cPCR using the B22/23 primers alone. These results could indicate a higher sensitivity of the B22/23 primer pair compared to the JW62/63 primers, similar to the study by Okay et al. (2009) who, on testing amniotic fluid samples, detected 9 (18.0%) more positive cases in nested PCR using the B22/23 primers than in cPCR (JW62/63).

These discordant findings between molecular methods have also been reported by other studies (Chabbert et al., 2004; Hierl et al., 2004; Nagy et al., 2006; Okay et al., 2009). These authors suggest that disagreements may be related to various factors such as the methods used for genomic DNA extraction, removal of PCR inhibitory factors, the choice of primers and amplification parameters (Chabbert et al., 2004; Nagy et al., 2006; Okay et al., 2009).

Only one sample with low IgG avidity (1.9%) was positive by qPCR. This finding is significant, because it is expected to detect parasitism in cases of acute infection (Yamada et al., 2011), as was observed for this sample by low avidity and a positive result by PCR in amniotic fluid.

The results of the PCRs in respect to the avidity test showed that 5 positive samples (nested PCR = 3; cPCR (B22/23) = 4) had high avidity, and one sample positive by qPCR had low avidity. Given these findings, it is not possible to rule out the possibility of false-positive results by nested PCR and cPCR, as the serological results are suggestive of chronic infection, under which conditions it is not expected to detect parasitism. Teixeira et al. (2013) described 4 false-positive cases by nested PCR using the B22/23 primers to test amniotic fluid samples. The false-positive results found by cPCR may be related to the fact that carryover associated with post-PCR handling may occur in cPCR and nested PCR with agarose gel electrophoresis and the re-amplification of the PCR product in the nested PCR (Lin et al., 2000; Teixeira et al., 2013). The results of qPCR are understandable as the result of serology suggests an acute infection, which is characterized by periods of parasitism (Cordeiro et al., 2010). Moreover, qPCR is considered by some authors as a more sensitive technique with low risks of contamination (Homan et al., 2000; Lin et al., 2000; Murat et al., 2013).

In G1, only one sample was positive by cPCR using the B22/B23 primer pair. This pregnant woman presented serologically nonreactive IgA and IgM antibodies but she had high concentrations of IgG antibodies by ELISA and ELFA. Cases of chronically infected pregnant women that had reactivation of the disease during pregnancy were reported by Andrade et al. (2010) and Olival et al. (2014), with transmission of the disease to their babies and by Avelar et al. (2015) with a case that progressed to stillbirth during the 34th week of pregnancy. Moreover, de Souza et al. (2015) reported seroconversion in the 17th week of gestation resulting in a spontaneous abortion. The current study only evaluated pregnant women and so serological and molecular monitoring of the mother and the baby would be necessary to confirm the suspicion of gestational and congenital infection. In any case, the possibility of a false-positive result was not ruled out using cPCR, since parasitism was not identified by other methods.

Of the 50 samples of amniotic fluid subjected to cPCR, 29 (58.0%) were positive, demonstrating the importance of amniocentesis to assist the diagnosis of fetal infection. When we compared the results of cPCR for amniotic fluid to PCR results for blood samples, we found a statistical difference (cPCR (B22/23): P < 0.0001; 95% CI: 0.3647–0.6753; nested PCR: P < 0.0001; 95% CI: 0.3895–0.6905 and qPCR: P < 0.0001; 95% CI: 0.4146–0.7054). Ivovic et al. describe several factors that may make the identification of parasite DNA in blood samples difficult, such as the short time the parasite remains in the blood and the action of the immune system that rapidly destroys parasites in the circulation, the small amount of blood drawn compared to the total volume of blood in the human body and PCR inhibition factors present in blood. Moreover, PCR of amniotic fluid samples has shown greater sensitivity compared to other methods such as bioassays and cell cultures (Toulon et al., 1999; Ivovic et al., 2012), thereby proving it to be an important test contributing to the diagnosis of gestational and fetal infections.

In our study, it was not possible to follow up the newborns and establish a relationship of maternal infection with the clinical aspects presented by the neonate in the recommended period, which is a limitation of this study (McLeod et al., 2014; Murat et al., 2013b; Robert-Gangeux and Belaz, 2016; Robert-Gangeux and Dardé, 2012).

In the current study, the differences between the results of the molecular methods highlight the real difficulties still found in the molecular diagnosis of toxoplasmosis using human samples. However, the results of the serological methods used in this study, ELISA and ELFA, agreed for both IgM and IgG antibodies, which is an important finding as these methods are widely used for screening and studies of anti-T. gondii antibodies in different populations of patients such as pregnant women, neonates and immunocompromised patients. The results showed concordance between the serological methods (ELISA and ELFA); however, ELFA identified more positive cases than ELISA. The molecular methods were discrepant with cPCR using the B22/23 primers having the highest sensitivity, but lower specificity compared to the other molecular methods.

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