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Prenatal and Early Postnatal Diagnosis of Congenital Toxoplasmosis in a Setting With No Systematic Screening in Pregnancy

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To determine the risk of congenital toxoplasmosis (CT) and provide early (pre- or postnatal) identification of cases of CT in the absence of systematic screening in pregnancy.

In the presented cross-sectional study, serological criteria were used to date *Toxoplasma gondii* infection versus conception in 80 pregnant women with fetal abnormalities or referred to as suspected of acute infection, and in 16 women after delivery of symptomatic neonates. A combination of serological, molecular (qPCR), and biological (bioassay) methods was used for prenatal and/or postnatal diagnosis of CT.

Most (77.5%) pregnant women were examined in advanced pregnancy. Of all the examined seropositive women (n = 90), infection could not be ruled out to have occurred during pregnancy in 93.3%, of which the majority (69%) was dated to the periconceptual period. CT was diagnosed in 25 cases, of which 17 prenatally and 8 postnatally. Molecular diagnosis proved superior, but the diagnosis of CT based on bioassay in 7 instances and by Western blot in 2 neonates shows that other methods remain indispensable.

In the absence of systematic screening in pregnancy, maternal infection is often diagnosed late, or even only when fetal/neonatal infection is suspected. In such situations, use of a complex algorithm involving a combination of serological, biological, and molecular methods allows for prenatal and/or early postnatal diagnosis of CT, but lacks the preventive capacity provided by early maternal treatment.

(*Medicine* 95(9):e2979)

Abbreviations: AF = amniotic fluid, BA = bioassay, CT = congenital toxoplasmosis, FB = fetal blood, HSDA = high sensitivity direct agglutination assay, ISAgA = immunosorbent agglutination assay, NB = neonatal blood, NPV = negative predictive value, NRLT = National Reference Laboratory for Toxoplasmosis, PPV = positive predictive value, qPCR = quantitative (real-time) PCR, *T.gondii* = *Toxoplasma gondii*, WB = Western blot.

Editor: Chia-Kwung Fan.
Received: November 11, 2015; revised: January 25, 2016; accepted: February 9, 2016.
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The study was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (project grant III 41019).
The authors have no conflicts of interest to disclose.
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ISSN: 0025-7974
DOI: 10.1097/MD.0000000000002979

INTRODUCTION

Toxoplasma gondii is a ubiquitous protozoan parasite estimated to infect one-third of the global population.[1,2] Although generally mild and self-limiting in immunocompetent individuals, *T. gondii* infection may cause life-threatening disease in the fetus and in the immunosuppressed host. Congenital transmission remains a considerable burden on global health.[3] Fetal infection may result in intrauterine death or clinical symptomatology at birth, or be subclinical at birth, but unless treated, visual or neurological sequelae may develop later in life.[4] This emphasizes the need for timely diagnosis and treatment.

Vertical transmission of *T. gondii* may occur following maternal primary infection, characterized by the presence of actively dividing parasites in the bloodstream of the pregnant woman. The risk of parasites reaching the fetus depends mostly on the gestational age at the time of infection, increasing from less than 10% in the first trimester, to 30% in the second and 70% to 90% in the third trimester,[5] but infection acquired up to 2 months before conception may also endanger the fetus.[6–13] This implies that for the assessment of fetal risk, dating of the infection versus conception is of the utmost importance. As infection is clinically manifested in not more than 10% of cases,[14] the diagnosis of infection in pregnancy relies on serology. As a general rule, detection of specific IgM and low avidity IgG antibodies indicates recent infection. However, serological findings, particularly if initially performed in late pregnancy, may be challenging to interpret. Even in the absence of specific IgG antibodies, detection of specific IgM requires further follow-up testing to exclude the possibility of early infection.[15] It is well known that specific IgM may last for prolonged periods of time,[16,17] and so can low avidity.[18–20] On the other hand, detection of high avidity IgG antibodies rules out primary infection within the last 4 months. This means that not even high avidity, if detected in late pregnancy, can rule out infection in early pregnancy or close to conception,[11–13,21] that is in the periconceptual period (2 months prior to or following the assumed date of conception).

Monthly serological screening of pregnant women allows for the time of seroconversion to be established within a margin of few weeks. However, systematic screening programs exist in only a limited number of European countries (France, Austria, Belgium, Norway, and part of Italy). In contrast, in an overwhelming majority of the world countries, maternal *T. gondii* infection in pregnancy may only be suspected based on fetal symptomatology, and is often diagnosed in advanced

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pregnancy. To date maternal infection, late testing requires additional refinement of the diagnostic criteria based on the expected kinetics of specific IgM and maturation of specific IgG avidity over time.

In Serbia, laboratory diagnosis is carried out in routine laboratories, and patients with discrepant or inconclusive results are referred to the National Reference Laboratory for Toxoplasmosis (NRLT) at the Institute for Medical Research in Belgrade. We present an NRLT-based study of prenatal and/or early postnatal diagnosis of CT in offspring of women suspected of toxoplasmosis in pregnancy and of mothers who have not been tested until after childbirth, using a standardized approach (reflected in a complex algorithm).

METHODS

Patients

A total of 96 women were enrolled in this prospective study between September 1, 2008 and August 31, 2014, of which 80 were tested during pregnancy and 16 after childbirth.

Inclusion criteria for serological testing of pregnant women included sonographically suspected or documented fetal malformations and/or suspected toxoplasmosis in pregnancy based on serological screening in routine laboratories. Women first tested after childbirth were included based on clinical manifestations suggestive of congenital infection in their neonates.

Among the 80 women tested in pregnancy, prenatal diagnosis of CT was conducted in 65 fetuses from 63 pregnancies (2 twin pregnancies). Of these, postpartal follow-up was continued in 23 neonates.

Postnatal diagnosis of CT was conducted in a total of 55 neonates. These included those 16 with clinical manifestations at birth, born to mothers who have not been tested during pregnancy, and 39 neonates born to mothers examined during pregnancy. Of the latter, 23 had been subjected to prenatal diagnosis, while the mothers of 16 were only tested serologically.

Biological samples for prenatal diagnosis included amniotic fluid (AF) in 60 and fetal blood (FB) in 5 cases, and for postnatal diagnosis neonatal blood (NB) samples in all 55 neonates.

The study was approved by the Institute for Medical Research Ethics Committee (EO103/2014).

Laboratory Methods

All biological materials including peripheral blood, AF, and FB samples were first centrifuged (2200 rpm for 15 minutes), and the supernatant was used for serology while the pellet (cell material) was used for molecular and biological analysis.

Serological screening of the women was performed on a VIDAS analyzer, using TXG-VIDAS ($<$4 IU/mL considered negative; 4–8 IU/mL borderline and $\geq$8 IU/mL positive) and TXM-VIDAS (results expressed as indices: $<$0.55 negative, 0.55–0.65 borderline, and $\geq$0.65 positive) (bioMérieux, Marcy l'Etoile, France) assays for specific IgG and IgM detection, while specific IgG avidity was determined by TXGA-VIDAS assay (results expressed as indices: $<$0.200 low, 0.200–0.300 borderline, and $\geq$0.300 high avidity) (bioMérieux).

Serology in FB and NB samples included in-house high sensitivity direct agglutination assay (HSDA)[22] for specific IgG (results expressed as titers; titer $\geq$1:40 considered positive), the antigen for which was kindly provided by Isabelle Villena, Reims, France, and the immunosorbent agglutination assay (ISAgA) (bioMérieux) for specific IgM and IgA (results expressed as indices on a scale from 0 to 12; results $\geq$3 considered to be indicative of CT).

Furthermore, neonatal and maternal serological profiles were compared using the *T. gondii* Western blot (WB) IgG/IgM test (LD-Bio Diagnostics, Lyon, France). According to the manufacturer's instructions, any well-defined band of a molecular weight $<$120 kDa present in the neonate but absent from the mother's serum indicates anti-*T. gondii* antibody neosynthesis by the neonate.

All commercial tests were performed according to the respective manufacturers' recommendations.

Molecular methods included DNA extraction using 200 μL of sample by QIAmp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and quantitative detection of the *T. gondii* 529 bp repetitive element (*AF146527* gene) by real-time PCR (qPCR), using the protocol we described elsewhere.[23]

Bioassay (BA) of biological samples in an attempt to isolate the parasite included intraperitoneal inoculation of 500 μL of sample pellet in 2 Swiss-Webster mice followed by microscopic examination of mice brain homogenates for *T. gondii* cysts and murine serology (HSDA) 6 weeks later. The detailed protocol was described previously.[24]

Diagnostic Criteria

The diagnosis of maternal infection was based on serology. The criteria for the interpretation of the serological results according to which the examined women were classified into the infection stages (as acute, subacute, or chronic) are presented in Table 1. To date maternal infection to the gestational age, these criteria were further refined to take into account the time elapsed between testing and the presumed time of infection (Table 2), based on the following: a positive IgM test, while not necessarily indicating recent infection, warrants further testing before a final conclusion can be made; the kinetics of IgG avidity maturation as shown by the study of Fricker-Hidalgo et al,[25] which showed that a VIDAS avidity index remains under 0.100 during the first 2 to 3 months, and an index of $\geq$0.600 is reached after 9 to 15 months.

The diagnosis of CT relied on direct diagnosis, that is isolation of the parasite or detection of its DNA, while serological findings generally served to complement the diagnosis.

TABLE 1. Serological Criteria for the Determination of the *Toxoplasma gondii* Infection Stage in Acquired Toxoplasmosis

Diagnostic Criteria			
IgG	**IgG Avidity**	**IgM**	***Toxoplasma gondii* Infection Stage**
Neg	/	Neg	Seronegative
Pos	Low	Pos	Acute
Pos	Border/high	Pos	Subacute
Pos	Low	Neg	Subacute
Pos	Border/high	Border	Subacute
Pos	High	Neg	Chronic

Border = borderline, IgG avidity = TXGA-VIDAS result for specific IgG avidity, IgG = TXG-VIDAS result for specific IgG, IgM = TXM-VIDAS result for specific IgM, Neg = negative, Pos = positive.

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TABLE 2. Criteria for the Timing of *Toxoplasma gondii* Infection Versus Conception in Pregnant Women and After Childbirth

	T. gondii Infection Dating Criteria			
First Tested	**IgM**	**IgG Avidity**	**Infection Stage**	**Time of Infection Versus Conception**
1st trimester	Neg	High	Chronic	>2 m before conception
	Pos/border	High/border	Subacute	Periconceptual cannot be excluded
	Pos	Low (0.100–0.200)	Acute	Periconceptual
	Pos	Low (<0.100)	Acute	1st trimester
2nd trimester	Neg	High	Chronic	>2 m before conception
	Pos/border	High	Subacute	Periconceptual cannot be excluded
	Pos	Border	Subacute	Periconceptual
	Pos	Low (0.100–0.200)	Acute	1st trimester
	Pos	Low (<0.100)	Acute	2nd trimester
3rd trimester	Neg	High	Chronic	>2 m before conception
	Pos/border	High	Subacute	Periconceptual
	Border	Border	Subacute	1st trimester
	Pos	Border	Subacute	2nd trimester
	Pos	Low (0.100–0.200)	Acute	2nd trimester
	Pos	Low (<0.100)	Acute	3rd trimester
After delivery	Neg	High (>0.600)	Chronic	>2 m before conception; considered of no risk in pregnancy
	Neg	High (0.300–0.500)	Chronic	>6 m before delivery but periconceptual cannot be excluded
	Border	High/border	Subacute	1st trimester
	Pos	High/border	Subacute	2nd trimester
	Pos	Low	Acute	3rd trimester

Border = borderline, m = months, Neg = negative, Pos = positive.

Figure 1 presents the algorithm used for the diagnosis of CT in the NRLT.

Statistical Analysis

Comparison of the methods used for the diagnosis of CT was performed using 2 by 2 contingency tables. Standard parameters of agreement including sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were determined. Results were statistically analyzed using the SPSS software, version 11.5 (SPSS Inc, Chicago, IL). The level of statistical significance was 5% ($P < 0.05$).

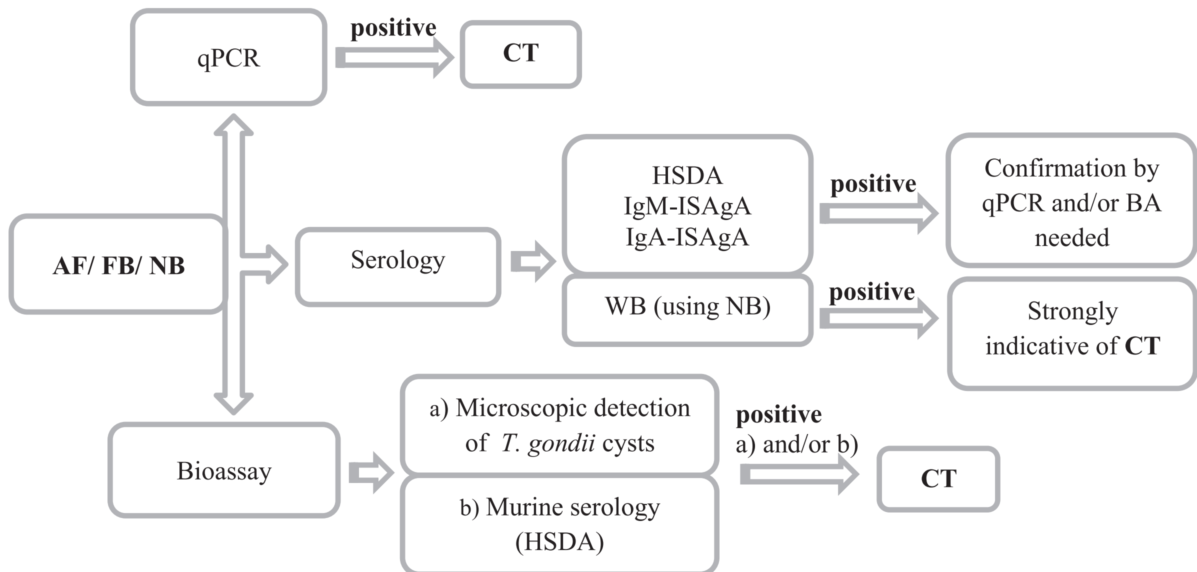

FIGURE 1. Algorithm for the diagnosis of congenital toxoplasmosis. AF = amniotic fluid; BA = bioassay; CT = congenital toxoplasmosis; FB = fetal blood; HSDA = high sensitivity direct agglutination assay; IgM-ISAgA = ISAgA test for detection of specific IgM, IgA-ISAgA = ISAgA test for detection of specific IgA, NB = neonatal blood; qPCR = quantitative PCR; WB = Western blot.

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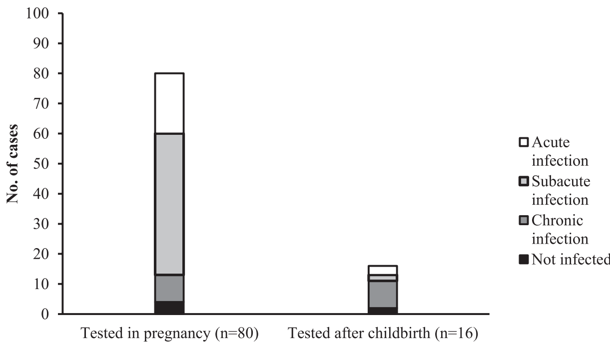

FIGURE 2. Stage of *Toxoplasma gondii* infection in the examined women as determined at the time of initial testing.

RESULTS

Of the 80 women tested during pregnancy, 22.5% were examined during the first, 61.3% during the second, and 16.2% during the third trimester. Only 7 (8.8%) reported lymphadenopathy. Of the 16 women tested because of symptomatic offspring, none reported clinical manifestations during pregnancy. All women were tested serologically for *T. gondii*-specific IgM and IgG antibodies as well as for IgG antibody avidity, and the results interpreted according to the criteria presented in Table 1. The stage of infection at the time of testing is presented in Figure 2, and of the time of infection in relation to conception for infected women in Figure 3. Of all the examined seropositive women (n = 90), infection during pregnancy could not be ruled out in 93.3%, of which the majority (69%) was dated to the periconceptual period.

All pregnant women diagnosed with acute or subacute toxoplasmosis were immediately started on spiramycin treatment. In case of confirmed fetal infection, treatment was switched to pyrimethamine-sulphadiazine and folinic acid until delivery, and continued during the entire first year of life of the neonate.

Prenatal Diagnosis of CT

Of the 80 women tested during pregnancy, 4 were shown to be noninfected and 3 to be infected long before conception; these 7 were obviously cases of erroneous diagnosis in a primary level laboratory. Out of the 73 women with proven or suspected infection in pregnancy, 63 (86.3%) opted for prenatal diagnosis of fetal infection.

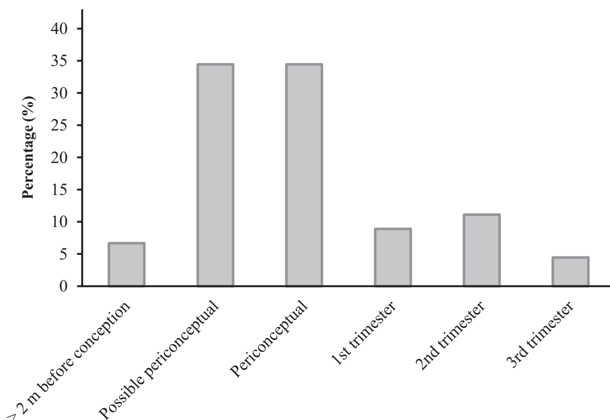

FIGURE 3. Dating of *Toxoplasma gondii* infection versus conception in seropositive women (n = 90).

Direct Diagnosis

Of the 65 prenatal samples, qPCR was performed in 54 (83.1%), of which *T. gondii* DNA was detected in 11 (20.4%). Bioassay was performed in 62 (95.4%), and viable parasites were isolated in 10 (16.1%). CT was diagnosed by qPCR alone in 7 (41.2%), by both qPCR and BA in 4 (23.5%), and by BA alone in 6 (35.3%, but in 4 of these qPCR was not performed), indicating a total of 17 (26%) fetal infections detected by direct methods. Complete findings in these 17 infected fetuses are presented in Table 3, with reference to estimated time of maternal infection versus conception. As presented, CT was an outcome of infection dated to the periconceptual period in 14 of 17 (82.4%) and to the second trimester in 3 of 17 (17.6%) cases.

Indirect Diagnosis

Specific IgM antibodies (index $\geq$3) were detected in 4 (6.1%) prenatal samples (3 AF and 1 FB sample), of which CT was confirmed in 2 (50%) (Table 3, cases 9, 10). Specific IgA antibodies were detected in 9 of 15 (60%) samples (8 AF and 1 FB sample), of which CT was proven by direct methods in 5 of 9 (55.6%) (Table 3, cases 5, 7, 8, 9, 14).

Early Postnatal Diagnosis and/or Follow-Up

Postnatal diagnosis of CT was performed in 55 neonates, of whom 32 (58.2%) were not examined previously. The other 23 (41.8%) were already examined prenatally, when 8 were diagnosed with CT.

Direct Diagnosis

Molecular diagnosis of CT was conducted in 41 (74.5%) neonates, and parasitic DNA was detected in 6 (14.6%). In 1 of these, the postnatal qPCR result only confirmed the prenatally established diagnosis (Table 3, case 10), but in the other 5, it determined the diagnosis of CT (Table 4). The results of postnatal diagnosis in infected neonates, along with the symptomatology, and results of maternal infection dating are presented in Table 4.

Of the 41 neonates tested by PCR, 34 mothers had been examined during pregnancy and prenatal diagnosis of CT had been performed in 20 (58.8%). Importantly, no PCR result obtained postnatally reversed any of the negative results of prenatal molecular diagnosis. In contrast, in 4 cases (Table 3, cases 13, 14, 15, 17), postnatal PCR was negative despite being positive prenatally. As 9 of 34 women tested during pregnancy did not undergo amniocentesis (upon gynecologist' advice or personal decision), qPCR was not performed until birth, when it detected 3 infected infants (Table 4, cases 6–8). Furthermore, detection of *T. gondii* DNA in NB was the earliest method of direct diagnosis for 13 of 16 (81.2%) neonates born to mothers first tested for toxoplasmosis after delivery, and identified another 2 infected children (2/13; 15.4%) (Table 4, cases 2 and 4).

Bioassay was performed in 20 (36.4%) neonates. Viable parasites were isolated in 7 (35%), including 5 (71.4%) neonates born to mothers examined during pregnancy and 2 (28.6%) neonates born to mothers tested after childbirth. In 3 of the 4 infected neonates of mothers tested during pregnancy (Table 3, cases 11, 13, 17), the bioassay result supported the prenatally

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TABLE 3. Prenatal Diagnosis of Congenital Toxoplasmosis, With Postnatal Follow-Up and Estimated Time of Maternal Infection

	Prenatal Diagnosis					Postnatal Diagnosis						
	Molecular		Conventional			Molecular		Conventional				
Case No.	qPCR (Ct)	*T. gondii* per mL	BA	IgM	IgA	qPCR (Ct)	*T. gondii* per mL	BA	WB	IgM	IgA	Estimated time of maternal infection
1	ND	NA	Pos	0	ND	/	/	/	/	/	/	Possible periconceptual
2	Pos (35.39)	31	Neg	0	ND	/	/	/	/	/	/	Possible periconceptual
3	ND	NA	Pos	0	ND	/	/	/	/	/	/	Possible periconceptual
4	ND	NA	Pos	0	ND	/	/	/	/	/	/	Possible periconceptual
5	Pos (34.30)	74	Neg	0	8	/	/	/	/	/	/	2nd trimester
6	Pos (35.30)	34	Pos	0	ND	/	/	/	/	/	/	Periconceptual
7	Neg	NA	Pos	0	11	/	/	/	/	/	/	Periconceptual
8	Pos (31.67)	588	Pos	0	4	/	/	/	/	/	/	Possible periconceptual
9*	Neg	NA	Pos	11	12	/	/	/	/	/	/	2nd trimester
10	Pos (36.96)	9	Neg	3	ND	Pos (38.9)	<1	ND	ND	ND	ND	Periconceptual
11	ND	NA	Pos	0	ND	ND	NA	Pos	ND	0	0	Possible periconceptual
12	Pos (36.72)	11	Pos	0	ND	ND	NA	ND	Neg	0	4	Periconceptual
13	Pos (34.60)	58	Neg	0	ND	Neg	NA	Pos	Pos	5	4	Periconceptual
14	Pos (35.76)	23	Neg	0	12	Neg	NA	Neg	Neg	0	12	Periconceptual
15	Pos (37.01)	13	Neg	0	0	Neg	NA	Neg	Neg	0	0	Possible periconceptual
16	Pos (36.48)	8	Neg	0	ND	ND	NA	ND	Neg	0	0	Possible periconceptual
17	Pos (29.96)	844	Pos	0	0	Neg	NA	Pos	Pos	0	5	2nd trimester

BA = bioassay, Ct = cycle threshold, IgA = ISAgA result for specific IgA, IgM = ISAgA result for specific IgM, NA = not applicable, ND = not done, / = not followed-up postnatally, Neg = negative, Pos = positive, qPCR = quantitative PCR, WB = Western blot.
* FB sample.

established diagnosis, but in 2 (Table 4, cases 6, 7) it was of diagnostic value (alongside qPCR).

Indirect Diagnosis

Specific IgM antibodies were detected in 7 (12.7%) neonates at birth, but clearance of specific IgM in subsequent samples in 1 (14.3%) case, along with the negative results of direct methods, confirmed that the detected antibodies were a result of contamination during delivery.[26] Importantly, all other 6 neonates in which specific IgM antibodies were detected were CT cases proven by direct methods (Table 3, case 13 and Table 4, cases 1–5).

Interestingly, in another 2 neonates specific IgM appeared months after birth, during the fifth (poor compliance with therapy, diagnosis established at birth) and fifteenth month of life (serological rebound upon completion of year-long treatment, CT diagnosed prenatally) (results not shown), respectively.

TABLE 4. Postnatal Diagnosis of Congenital Toxoplasmosis With Clinical Symptomatology and Estimated Time of Maternal Infection

	Direct Diagnosis			Indirect Diagnosis				
Case No	qPCR (Ct)	*T. gondii* per mL	BA	WB	IgM	IgA	Clinical Symptoms	Estimated time of maternal infection
1	Neg	NA	Pos	Pos	4	0	NIS, IUGR	3rd trimester
2	Pos (34.70)	20	Neg	Pos	12	12	NIS, IUGR	3rd trimester
3	ND	NA	ND	Pos	12	12	Hydrocephalus	> 6 months before delivery (Possible periconceptual)
4	Pos (37.26)	3	Pos	Pos	12	11	IUGR	3rd trimester
5	Neg	NA	ND	Pos	3	4	CNS abnormalities	> 6 months before delivery (Possible periconceptual)
6	Pos (34.45)	24	Pos	Pos	0	0	Asymptomatic	Periconceptual
7	Pos (36.86)	4	Pos	Pos	0	5	Asymptomatic	3rd trimester
8	Pos (36.63)	4	Neg	Neg	0	0	Asymptomatic	Periconceptual

BA = bioassay, Ct = cycle threshold, IgA = ISAgA result for specific IgA, IgM = ISAgA result for specific IgM, IUGR = intrauterine growth restriction, NA = not applicable, ND = not done, Neg = negative, NIS = nonspecific infectious symptomatology, Pos = positive, qPCR = quantitative PCR, WB = Western blot.

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Specific IgA antibodies, examined in 36 (65.5%), were detected in 13 (36.1%) neonates, of which CT was proven in 9 (69.2%) (Tables 3 and 4).

WB test was performed in 41 (74.5%) neonates. Among the 16 neonates diagnosed with CT that were available for postnatal diagnosis, WB was performed in 14, and neosynthesis of anti-*T. gondii* antibody was demonstrated in 9 (64.3%) (Tables 3 and 4). Of these, 7 were cases of CT diagnosed by direct methods, whereas in 2 cases of clinical infection a positive WB was the main diagnostic marker (Table 4, cases 3 and 5). An illustrative example of a positive WB is presented in Figure 4.

In total, CT was postnatally diagnosed in 8 neonates (Table 4). In addition, CT was postnatally confirmed in 6 of the prenatally diagnosed cases, of which in 4 by direct methods and in 2 only serologically (Table 3, cases 10–14, 17).

Test Comparison

Sensitivity, specificity, PPV, and NPV for each of the methods used for the diagnosis of CT are presented in Table 5. The sensitivity and NPV of PCR (80% and 91%, respectively) exceeded that of bioassay (41.7% and 51.6%, respectively), but were closely followed by WB (64.3% and 83.9%). Of all the indirect methods used for the diagnosis of CT, the specificity of WB was the highest and indeed equal to that of qPCR and BA (100%).

DISCUSSION

The main prerequisite for evaluating the risk of fetal *T. gondii* infection is precise timing of maternal infection versus conception, which is hampered by the lack of systematic screening. In this series, we tested women in various stages of pregnancy and/or after childbirth. The majority of pregnant women were tested in the second trimester (61.3%), and the infection was staged as subacute in 58.8%. This is not surprising given that only few (8.8%) reported lymphadenopathy in the preceding weeks or months.

Application of a set of criteria based on the kinetics of specific IgM and maturation of IgG avidity could not rule out periconceptual infection in even 69% women. Periconceptual infection is hypothesized to result from delayed parasite migration from the placenta to the fetus,[8,13] weeks and even months postinfection in the mother, a phenomenon recognized decades ago as ‘‘prenatal incubation period.’’[27] Long considered to be of low risk for transplacental transmission of the parasite, resulting in CT in under 2% of the cases,[28] recent studies reported transmission rates after periconceptual infection of up to 3.3% and 3.8%.[29,21] However, the 29% cases of CT resulting from periconceptual infection is striking. This result may be attributed to the study sample that, on the one hand, included a relatively high proportion (25%) of women included because of fetal/neonatal symptomatology, and on the other, a relatively high proportion of women (30%) tested late (in the 3rd trimester of pregnancy) or after childbirth, when the dating of maternal infection could not be more precise and may have resulted in an overestimation of infection dated to the periconceptual period.

Overall, using a complex algorithm for the diagnosis of CT which included molecular, biological, and serological methods, 25 cases of CT were diagnosed in this series. Of these, 17 were diagnosed prenatally, in cases of proven or suspected infection during pregnancy or the periconceptual period, while 8 were diagnosed after birth.

Analysis of the relative contribution of the direct diagnostic methods showed that fetal infection was diagnosed by an optimized qPCR protocol in 85% of the cases where performed (11/13), of which it was further supported in 4 (of the 11 PCR-positive cases, 36%) by isolation of viable parasites. On the other hand, in addition to these 4, bioassay was positive in another 2 cases in which PCR was negative (of 13, 15%; Table 3, cases 7 and 9) and in another 4 (where it was the only direct test performed; Table 3, cases 1, 3, 4, and 11). Thus, molecular diagnosis was essential in 7 (54%) fetuses in which *T. gondii* was not isolated by bioassay. However, failure to detect *T. gondii* DNA in 2 cases of parasite isolation may be explained by a 5-fold higher sample volume inoculated into mice than used for DNA extraction. In 1 of these cases, the material for molecular diagnosis was FB, known for high concentrations of PCR inhibitors which may result in false-negative qPCR. In the context of these real-life situations, although time-consuming (5–6 weeks needed for the result), bioassay remains indispensable in prenatal diagnosis of CT.[30]

Possible reasons for false-negative prenatal diagnosis include an inadequate time distance between maternal infection and amniocentesis. AF is generally sampled from the 16th gestational weeks onward and at least 4 weeks after detectable maternal seroconversion. However, this is challenging in the absence of systematic screening and amniocentesis may be performed too early or too late after maternal infection. False-negative results are quite common in early pregnancy seroconverters, due to very few cells and miniscule number of parasites in AF.[31,32] The number of parasites in AF samples detected by PCR in our study was rather small, below 100/mL in 9 of 11 positive samples, and 88.9% (8/9) of those samples originated from periconceptually infected women. Maternal treatment and/or delayed transplacental transmission could cause the parasites, if at all detectable in AF of periconceptually infected women, to be present in a small number.[8,10,33]

As for fetal serology, specific IgM were detected in 11.7% (2/17), while specific IgA were detected in 29.4% (5/17) of the infected fetuses, where they obviously reflected the fetal immune response to *T. gondii* infection. On the other hand, specific IgM were detected in 2 and specific IgA antibodies in

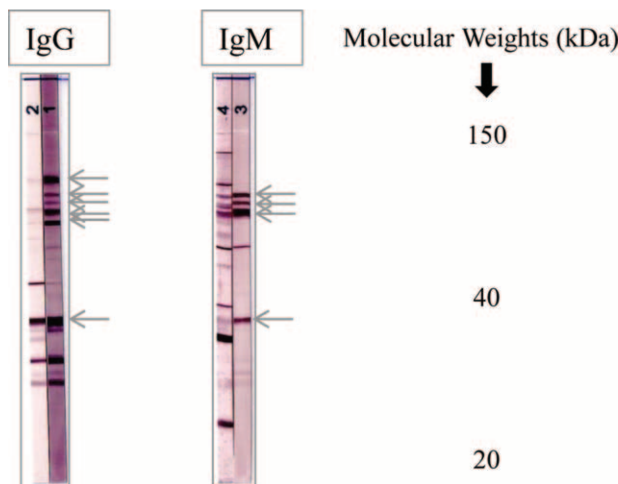

FIGURE 4. Representative example of Western blot test showing fetal neosynthesis of *Toxoplasma gondii*-specific antibodies at birth (arrows pointing out to the ‘‘new’’ bands). Left IgG/IgM strip: maternal serum; right IgG/IgM strip: neonatal serum.

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TABLE 5. Performance Characteristics of the Methods Included in the Diagnostic Algorithm for Congenital Toxoplasmosis

	Diagnostic Method/Assay				
Assay Characteristic	**qPCR**	**Bioassay**	**WB**	**IgM-ISAgA**	**IgA-ISAgA**
Sensitivity (%, 95% CI)	80 (64.5–80)	41.7 (29–41.7)	64.3 (43.8–64.3)	37.5 (19.6–43.4)	56.3 (35.6–71.6)
Specificity (%, 95% CI)	100 (92.4–100)	100 (84–100)	100 (88.9–100)	97.4 (90.1–99.9)	80 (63.5–92.3)
PPV (%, 95% CI)	100 (80.6–100)	100 (69.6–100)	100 (68.1–100)	85.7 (44.7–99.2)	69.2 (43.8–88.1)
NPV (%, 95% CI)	91.1 (84.2–91.1)	57.6 (48.4–57.6)	83.9 (74.6–83.9)	79.2 (73.2–81.1)	69.6 (55.2–80.2)

NPV = negative predictive value, PPV = positive predictive value, qPCR = quantitative PCR, WB = Western blot.

10 fetuses in which the diagnosis of CT was ultimately excluded. It has been suggested that specific IgA antibodies in the AF may be of maternal origin, as a consequence of passage due to increased placental permeability.[34]

Among the 55 studied neonates, CT was diagnosed prenatally in 8, while in another 8, CT was first diagnosed after birth. Of the latter, the diagnosis was based on PCR in 5 (of the 7 in which it was performed, 71.4%) and on bioassay in 1 (of 8, 12.5%). In 2 neonates (25%), however, it was based on the results of indirect methods (WB, ISAgA) only (Table 4, cases 3 and 5); notably, both had severe CNS abnormalities.

NB is not considered an ideal material for molecular diagnosis of CT because, if the infection was transmitted long before delivery, parasitemia is rarely detectable in the neonate, especially if the mother was treated during pregnancy.[35] However, it allowed us to diagnose CT in 2 neonates with very low parasitemia and negative bioassay (20 and 4 parasites/mL, respectively). Generally, the parasitic load determined by qPCR was remarkably low, ranging from 3 to 24 parasites/mL. Bearing in mind that the sensitivity of bioassay is 10 cysts/mL, the low number of parasites in the above cases may have easily remained undetected by conventional methods of parasite isolation. Overall, the qPCR protocol applied in this study had a 91% NPV and 80% sensitivity, nearly twice as high as the sensitivity of bioassay (41.7%).

Specific IgM and IgA antibodies were detected in 37.5% (6/16) and 56% (9/16) of infected neonates, respectively. Previous studies have shown that specific IgM and IgA are usually detected in neonates after third trimester infections,[36] which accounted for only 7.8% of the postnatal diagnoses in our series (Figure 3). More frequent detection of specific IgA than specific IgM antibodies in infected neonates born to women infected in early pregnancy has been reported.[37]

WB is the most reliable serological marker of CT.[18,38,39] Of the neonates with positive WB in our series, specific IgM and specific IgA antibodies were both undetectable in 11.1% (1/9), and alone in another 22.2% (2/9) and 11.1% (1/9), respectively. The WB result supported the diagnosis of CT in 7 of 8 (87.5%) postnatally diagnosed neonates. The specificity of WB paralleled that of the direct methods (100%); furthermore, it was the crucial diagnostic marker in 2 neonates, in 1 of which PCR was negative while direct diagnostic methods were not performed in the other. However, WB was negative in 5 infected neonates, all born to periconceptually infected mothers (Table 3, cases 12, 14–16; Table 4, case 8). In these neonates, the diagnosis was based on positive qPCR, lending further support to the superiority of molecular over serological diagnosis in early pregnancy or periconceptual infection.

Although this study did not aim to analyze treatment effects, it should be noted that no (additional) clinical manifestations were observed at birth in any of the diagnosed and treated CT cases, as opposed to clinical CT in unrecognized cases of maternal infection during pregnancy (Table 4, cases 1–5). This further emphasizes the significance of early diagnosis.

In conclusion, early diagnosis of maternal and subsequent fetal *T. gondii* infection is a prerequisite for early treatment. This is best achieved by systematic screening in pregnancy, a practice unfortunately limited to very few countries. In the rather universal situation of a no screening in pregnancy policy, the results of this study show that the use of a combination of serological, biological, and molecular methods to date maternal infection and diagnose CT may help equalize the opportunities for a *T. gondii*-free life to offspring regardless of the national health policy. This approach, however, still lacks the preventive capacity provided by early maternal treatment and is associated with increased costs and unnecessary anxiety in a number of pregnant women while awaiting the results and outcome.

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