A novel interpretation of molecular diagnosis of congenital toxoplasmosis according to gestational age at maternal infection

Running Title: Molecular diagnosis of congenital toxoplasmosis

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Abstract

From a prospective cohort of 344 women who seroconverted for toxoplasmosis during pregnancy, 344 amniotic fluid, 264 placenta and 216 cord blood samples were tested for diagnosis of congenital toxoplasmosis using the same PCR assay. Sensitivity and negative predictive value of the PCR assay using amniotic fluid were 86.3% and 97.2%, respectively, and both specificity and positive predictive value were 100%. Using placenta and cord blood, sensitivities were 79.5% and 21.2%, and specificities 92% and 100%, respectively. In addition, the calculation of pre-test and post-test probabilities and the use of logistic regression allowed us to obtain curves that give a dynamic interpretation of the risk of congenital toxoplasmosis according to gestational age at maternal infection, this being for the three sample types (amniotic fluid, placenta and cord blood). Two examples are cited here: for a maternal infection at 25 weeks of amenorrhea, a negative result of prenatal diagnosis allowed estimating the probability of congenital toxoplasmosis at 5%, instead of an a priori (pre-test) risk at 33%. For an infection at 10 weeks of amenorrhea associated with a pre-test congenital toxoplasmosis risk at 7%, a positive PCR result using placenta at birth yields a risk increase to 43%; while a negative result damps down the risk to 0.02%. Thus, using a highly-performing molecular diagnosis, and in spite of the persistence of false negatives, post-test risk curves using both negative and positive results prove highly informative, allowing a better assessment of the actual risk of congenital toxoplasmosis and finally an improved decision guide to treatment.
Introduction

Toxoplasmosis is a worldwide infection due to the protozoan parasite *Toxoplasma gondii*, which is generally benign, but may cause severe infections in the fetus and the immunocompromised patient. A fetus can become infected and develop congenital toxoplasmosis if the mother contracts toxoplasmosis during pregnancy (7). For the pregnant woman, as for in otherwise healthy adults, the parasite rarely causes any symptoms. On the contrary, congenital toxoplasmosis has a wide clinical spectrum varying from lethal forms to subclinical forms yet likely to remotely produce ocular lesions that may eventually lead to blindness (27). The risk of transmission to the fetus, hence of congenital toxoplasmosis, increases, but its severity decreases, with gestational age at maternal infection (15). In France, in pregnant women aged 20-40 years old, the rate of infection by *T. gondii* presently varies from 6.1 to 7.2 per 1000; and approximately 300 newborns annually suffer from congenital toxoplasmosis (4, 22, 41). Early diagnosis of congenital toxoplasmosis in pregnancy is crucial for proposing the most appropriate therapeutic approach in order to prevent late complications. In France and in a few European countries where toxoplasmosis is regarded as a serious public health problem, a screening and monthly serologic follow-up of non-immune pregnant patients are established in order to reduce the frequency of congenital toxoplasmosis (2, 39, 41). A network of public regional reference laboratories exists throughout France, to which private laboratories may send their sera if the interpretation is difficult: e.g. to confirm the specificity of low IgG or IgM titers, or to date the infection using IgG titer kinetics or the avidity index of *Toxoplasma*-specific IgG. This proficiency network was formally organized with the creation of the National Reference Centre for Toxoplasmosis in 2006 (22) (https://www.chu-reims.fr/professionnels/cnr-toxoplasmose-1).
The diagnosis of congenital toxoplasmosis may prove a difficult task, combining clinical features and results of a battery of serologic and molecular tests. A combination of criteria was defined by the European research Network on Congenital Toxoplasmosis (23), in order to set up a 'gold standard' for the diagnosis of this disease in Europe. Different time steps in the microbiological diagnosis of congenital toxoplasmosis, using different methods on different samples, may be individualized: during pregnancy, at birth and during the first year of age. First, serologic tests are able to detect toxoplasmosis acquired by the mother during gestation. Accurate determination of the date of maternal infection is essential to evaluate the fetal risk for developing congenital toxoplasmosis (14). This is achieved when seroconversion is observed, or by using the kinetics of specific IgG in successive serum samples and the calculation of the IgG avidity index (reviewed in (28)). Then, depending on the term, prenatal diagnosis (PND) may be proposed to pregnant women experiencing primary infection with *T. gondii*: in France, this includes a molecular test using amniotic fluid and/or monthly ultrasonography examinations. At birth, molecular tests can also be performed using placenta (5, 17, 18, 29) and cord blood. Finally, at birth and during follow-up, serologic tests can confirm or rule out the diagnosis of congenital toxoplasmosis. A one-year follow-up of the infant is recommended before definitely ruling out the diagnosis of congenital toxoplasmosis (23).

The PND of congenital toxoplasmosis has been based on PCR using amniotic fluid since the 1990's, when it superseded former methods based upon *Toxoplasma* isolation in fetal blood and amniotic fluid by mouse inoculation, as well as the detection of specific IgMs and IgAs in fetal blood *in utero* (reviewed in (3), and (25, 26)). However, most PCR assays worldwide have remained 'in house' or 'laboratory-developed' assays, leading to a great heterogeneity in laboratory practices and methods (37) as well as to large variations in diagnostic performances (19, 36, 38). In congenital toxoplasmosis, the sensitivity and specificity of PCR
on amniotic fluid were reported to range from 40% to 100% and from 80 to 100%, respectively (reviewed in (3), (5, 33, 38, 43)). For molecular methods using placenta or cord blood, variable efficacies have been reported (reviewed in ref. (3) and (31)). It should be emphasized that molecular methods used for this diagnosis need to be highly specific but also highly sensitive since the parasite burden is often very low (12, 32); and both false positive and false negative results may lead to a wrong therapeutic decision in a critical situation.

In the current work, we aimed at determining the accuracy of the diagnosis of congenital toxoplasmosis using an optimized PCR assay on amniotic fluid, placenta and cord blood, in a large prospective cohort of 344 patients who successively underwent amniocentesis and whose children were followed-up after birth during at least one year. We analyzed the results of PCR tests according to gestational age at maternal infection and inferred accurate post-test predictive values for diagnosis. The final objectives of this work are to give practical up-to-date information with respect to disease risk assessment according to age at maternal infection, whether the molecular diagnosis of congenital toxoplasmosis is negative or positive.

Patients and Methods

Mother and child management. From January 1996 to December 2006, 344 women who contracted toxoplasmosis during pregnancy and underwent amniocentesis for molecular PND of congenital toxoplasmosis were enrolled in the study. The recruitment was regional (Languedoc-Roussillon, France). All Toxoplasma infections contracted during pregnancy and diagnosed according to state of the art serology were retained. Amniotic fluid sampling was performed after obtaining the written approval of the mother and, as recommended by the National Reference Centre for Toxoplasmosis, at least one month after the maternal infection for PND. Mothers were treated orally with 3 gr or 9 million U of spiramycin per day while
waiting for the results of PND. In addition, in order to detect possible abnormalities, an ultrasound scan was performed monthly. Signs suggestive of congenital toxoplasmosis were neurological abnormalities such as dilatation of the lateral ventricles or intracranial calcifications. In case of positive PCR result and abnormal cerebral findings, and according to parents’ opinion, termination of pregnancy was performed after expert assessment by a prenatal diagnosis multidisciplinary board. Pregnancies with positive PCR result and normal ultrasound findings continued until term with a treatment associating sulfadoxine and pyrimethamine and a rescue with folinic acid. At birth, neurological examination, ophthalmoscopy and transfontanellar cranial ultrasonography were performed on the newborn. All neonates and, when pregnancies were terminated, products of conception were included in our study. All biological tests were performed in the Parasitology-Mycology Department of the Montpellier University Hospital, France.

**Serologic tests.** The following techniques were used for detection of specific IgGs: direct agglutination assay Toxoscreen DA (Biomérieux®, France), indirect immunofluorescence (Biomérieux®, France), MEIA (microparticle enzyme immuno assay) Toxo-IgG EIA-kit (Axsym Abbott®, France); and for that of specific IgMs: indirect immunofluorescence (Biomérieux®, France), MEIA Toxo-IgM EIA-kit (Axsym Abbott®, France) and ISAGA (immunosorbent agglutination assay) (Biomérieux®, France). Dating the maternal infection was done either after serologic conversion, *i.e.* the shift from a negative to a positive *Toxoplasma* serology with specific IgMs and IgGs, or after studying the kinetics of specific IgG titer and taking into account the result of the test for Avidity of *Toxoplasma*-Specific Immunoglobulin G (BioRad®, France). At birth and during child follow-up, comparative maternal-child serology was performed with detection of specific IgGs, IgMs and IgAs. Detection of specific IgGs was also used to measure the mother to child ratio of *Toxoplasma*-specific IgG (so-called immune charge (IC)) [calculated by the following formula: IC =
Cmother/Cchild, where Cmother = anti-*T. gondii* IgG titer/total IgG concentration within the serum of the mother and Cserum represents the same ratio within the serum of the child] (13). The serologic surveillance of the child was continued during the first year of life until confirmation or exclusion of congenital toxoplasmosis.

**Parasite detection.** *Toxoplasma* was detected in amniotic fluid, placenta and cord blood by PCR and by inoculation into mice. The PCR detection of *Toxoplasma* DNA was performed routinely, using primers B22-B23 (8, 11) to amplify a fragment of the B1 gene (9). The assay used here was assessed in several studies as highly-performing, as judged from its sensitivity and specificity, and no modification of sensitivity was observed throughout the period of the study (11, 36). The assay was able to detect 0.5 tachyzoite per reaction, or 2.5 tachyzoites/mL of amniotic fluid (11, 36). For each amniotic fluid sample, a 4 mL-aliquot was processed fresh for DNA amplification using a method based on selective lysis of contaminating red blood cells, heat-detergent extraction and thermolysis (20). Placentas (300g) were ground, trypsinized, filtered, washed and lysed with proteinase K (Euromedex®, France); buffy coat portions (300 µL), isolated from cord blood after centrifugation 10 min at 1750 g, were also lysed with proteinase K (Euromedex®, France). Their DNA was prepared with the protein precipitation solution kit (A795A, Promega®, France). In all cases, the PCR was performed using 5 µL of the extracted DNA (11). According to good practices, negative controls, positive controls, search for inhibitors and a decontamination step with uracil-DNA-glycosylase to prevent carryover contaminations were performed in all reactions. As regards mouse inoculation, 500 µL of amniotic fluid, or placenta and cord blood were tested using the reference method in France (14). Briefly, half of the placenta preparation described above was injected intraperitoneally into five mice (1 mL per mouse); and the whole of the ground cord blood clot was inoculated into three mice (1 mL per mouse). Four and eight weeks after inoculation, blood samples were examined using an agglutination test (Toxo-Screen DA,
Biomerieux®, France) to detect the presence of *Toxoplasma*-specific IgG antibodies. Brains of seropositive mice were microscopically checked for the presence of *T. gondii* cysts to affirm infection.

**Case definition of congenital toxoplasmosis.** The criteria used in the present study to affirm or rule out the diagnosis of congenital toxoplasmosis (*i.e.* reference diagnosis) were those defined by the European research Network on Congenital Toxoplasmosis (23), that included serologic tests and mouse inoculation and were complemented by the results of the immune charge (13). These criteria represent the present 'gold standard' for diagnosing congenital toxoplasmosis in Europe. All cases were allocated into three mutually exclusive categories: (i) definite fetal infection; (ii) unlikely to have fetal infection; (iii) not infected. Due to the long term follow-up of children, inclusion in the first or in the latter category leaves no room for doubt and it was considered definitive. For this study, all definite congenital toxoplasmosis cases were confirmed after birth (or directly from aborted fetus), when at least one of the following criteria was met (23):

- detection of *Toxoplasma gondii* in amniotic fluid, or in cord blood by mouse inoculation,
- rise in IgG titers within the first year of life or persistently positive after that,
- positive specific IgMs and/or IgAs during the first six months of life,
- immune charge (see definition above) of the neonate three-fold higher than that of the mother.

It is noteworthy that the post-natal follow-up constitutes the basis for definitely affirming this diagnosis. Here, all cases of congenital toxoplasmosis diagnosed by PCR have been confirmed by one of the above criteria. A child was considered as not infected when disappearance of transmitted maternal IgG was documented by negative serology within the first year of life without treatment. On the other hand, cases were considered as 'unlikely to
have congenital toxoplasmosis’ when the evidence was suggestive but incomplete: i.e. when a drop in IgG titers were verified, but no follow-up was possible. In case of fetal loss, the diagnosis was assessed from ultrasonography findings and results of mouse inoculation and of pathological examination. It is noteworthy that for this study, the results of molecular diagnosis alone were not used to allocate a case in a category.

**Statistical analysis.** Sensitivity, specificity, positive (PPV) and negative predictive values (NPV), as well as positive and negative likelihood ratios, were calculated for PCR results according to gestational age at maternal infection. Likelihood ratios provide a direct estimation of how much a test result will change the odds of having a disease. The 95% confidence limits (CL95%) of sensitivity, specificity, PPV and NPV were computed using the binomial exact method or approximate normal formula. The confidence limits of LRs were computed with the log method (35). To estimate the pre-test probability (prevalence) and post-test probability (after the PCR results) of having congenital toxoplasmosis according to gestational age at maternal infection, a logistic regression model was used. Prevalence was estimated in the entire cohort. Post-test probabilities of congenital toxoplasmosis were estimated independently in the PCR-negative and PCR-positive populations: they correspond to 1-NPV and PPV, respectively. These results were reported on curves, where the values of prevalence and post-test risks of congenital toxoplasmosis were plotted against gestational age at maternal infection. The statistical software SAS Enterprise Guide (1) version 4.39 was used for statistical analysis.

**Results**

**Clinical and biological features of the cohort.**

Over an eleven-year period, 344 women who contracted toxoplasmosis during pregnancy and underwent amniocentesis for molecular PND of congenital toxoplasmosis in the Montpellier
University Hospital (France) were prospectively enrolled in this study (Fig. 1). Their children were then followed-up for a maximum period of time in order to affirm or rule out congenital toxoplasmosis. The demographic features of the cohort are shown in Supplemental Material (Table S1). The dates of infection (according to gestational age) were accurately established using serologic tests (see Patients and Methods) for 336 (97.7%) patients (Supplemental Material Table S2). In the entire survey, we had 46 cases (13.4%) lost to follow-up; 16 (4.7%) were lost before delivery and 30 (8.7%) during the postnatal period. Out of the 344 cases, 51 cases of congenital toxoplasmosis were diagnosed in 14 fetuses and 37 live-born children, yielding a global transmission rate of 14.8%. Symptomatic forms were seen in only 5.8% (20/344) of all pregnancies (Supplemental Material Table S2), all of them showing a positive PCR using amniotic fluid during PND. The outcome of all pregnancies is presented in Fig. 1. Fourteen pregnancies were terminated, 8 in the first and 6 in the second trimester. Among the 37 live-born children with congenital toxoplasmosis, only six presented a clinically symptomatic form (one case of intracranial calcifications, three cases of unilateral macular chorioretinitis and two cases of neuro-ocular cases with intracranial calcifications and unilateral macular chorioretinitis); 24 presented a subclinical form of the disease.

**Overall performances of amniotic fluid testing and dynamic interpretation of PCR results according to gestational age at infection.**

The performances of the different tests used in the study have been calculated based on all the biological results at the end of follow-up, and according to the 'gold standard' criteria defined by the European research Network on Congenital Toxoplasmosis (23). The detection of *Toxoplasma* DNA by PCR in amniotic fluid was positive in 44 out of the 344 samples and falsely negative in 7 out of the 51 congenital toxoplasmosis cases. The overall performances of molecular PND, confirmed by comparison with other tests, were as follows: sensitivity
86.3%, specificity 100%, PPV 100% and NPV 97.2% (Table I). Mouse inoculation of amniotic fluid was performed in 18 out of these 44 cases and found positive in eight cases, yielding a sensitivity of 44.4%; the specificity was 100% (Supplemental Material Table S3). In addition, logistic regression analysis allowed us calculating the 'pre-test' and 'post-test' risks of congenital toxoplasmosis, according to gestational age at infection and PCR results using amniotic fluid (see Materials & Methods and legend of Figure 2). As expected, a positive PCR on amniotic fluid was always associated with a 100% probability of giving birth to an infected child: for a maternal infection around 10 WA, the pre-test risk of congenital toxoplasmosis is only 7% (Fig. 2A, prevalence curve); however, if the PCR using amniotic fluid is positive, this risk obviously rises to 100% (Fig. 2A, PPV curve). More interestingly, Figure 2A also shows the usefulness of negative PCR results. For example, for an infection at 25 WA, the risk appears reduced from 33% (Fig. 2A, prevalence curve) to 4.8% (Fig. 2A, 1-NPV curve) if one takes into account the negative result; for a late infection (37 WA), a negative PCR result on amniotic fluid makes the risk fall from a pre-test 68% to a post-test value <20%. Pre- and post-test risk values can be extrapolated from the curves for any maternal infection date (Fig. 2).

Overall performances of placenta testing and dynamic interpretation of PCR results according to gestational age at infection.

The overall sensitivity of PCR using placenta was 79.5% (31/39) and its specificity was 92.4% (209/227). These figures were 70.7% (29/41) and 98.6% (236/239) for mouse inoculation using placenta (Supplemental Material Table S3). Here again, we applied logistic regression analysis for a dynamic view using placenta PCR results (Fig. 2B): for example, for a maternal infection at 15 WA, the pre-test probability of having congenital toxoplasmosis is 12.5%, but if the placenta is PCR-negative, the post-test risk is 0.8%; this figure rises to 53% if the PCR is positive. For a maternal infection at 20 WA, the figures for the prevalence, on
the one hand, and the risk estimates in case of a negative and a positive PCR result, on the other hand, are 21%, 3% and 66%, respectively; and at 25WA: 33%, 9% and 74%, respectively (Fig. 2B).

**Overall performances of cord blood testing and dynamic interpretation of PCR results according to gestational age at infection.**

Using cord blood at delivery, the sensitivity of the PCR assay was 21.2% (7/33) versus 8.8% using mouse inoculation; specificity was 100% (183/183 and 186/186) for both techniques (Table I, Supplemental Material Table S3). As for amniotic fluid and placenta, one can determine the prevalence and post-test risks in case of a negative and positive PCR: for example, these are 21%, 3% and 66%, respectively, for an infection at 20 WA, and 33%, 9% and 74%, respectively, for 25 WA (Fig. 2C).

**PCR testing of amniotic fluid, placenta and cord blood in the same patients.**

In our cohort of 344 patients, all three samples (amniotic fluid, cord blood and placenta) were assayed by PCR in 213 cases, of which 199 had a complete follow-up; 34 out of the 37 live-born congenital toxoplasmosis cases had the three samples tested (Supplemental Material Table S4). All three samples were PCR-positive in five cases only (15% of the 37 congenital toxoplasmosis cases); the dates of maternal infection for these cases were 4, 10, 24 30 and 31 WA. Amniotic fluid was the only PCR-positive sample in six cases (18%); the dates of maternal infection were 23 and 25 WA in two cases and 31 WA in the remaining six cases. Conversely, in only two cases, both placenta and cord blood were found PCR-positive whilst amniotic fluid was negative; the dates of maternal infections for those two cases were 18 and 24 WA; and the dates of PND were 24.5 and 28.5 WA, respectively. In one case of congenital toxoplasmosis, the three molecular tests were negative. Thus, the rate of false negative results for molecular diagnosis as a whole (using the three samples) was as low as 3%.

**Serologic diagnosis and follow-up.**
‘State of the art’ serology was used during pregnancy for dating maternal infection and in the child for assessing the definite status of the *Toxoplasma* infection; thus all congenital toxoplasmosis cases diagnosed by molecular methods were confirmed at birth or during follow-up by serology. The details of this are shown in Supplemental Material Table S2.

Serology in the neonatal period was contributive for the diagnosis of congenital toxoplasmosis in 37 cases: it was positive and confirmed the positive molecular PND in 30 cases; but it was also positive in seven false negative results of PND, allowing the diagnosis to be made. In the remaining 14 congenital toxoplasmosis cases, a termination of pregnancy occurred and serologic testing was not possible. The sensitivity of IgM and IgA detection at birth was 62.2% (23/37) and 59.5% (22/37), respectively. A positive immune charge of specific IgGs (see Material and Methods for definition) was found in 6/37 (16.2%) cases; one of these had a negative molecular PND, and was diagnosed with this only parameter.

**Discussion**

Our study shows that an optimized PCR assay can detect 86% of congenital toxoplasmosis cases prenatally, therefore leaving only 14% undetected at this stage. We also show the interest of molecular testing of the placenta and cord blood at birth for this diagnosis. This interest is highlighted by the novel dynamic analysis of the risk of congenital toxoplasmosis that we propose, taking into account not only the classical gestational age at maternal infection but also the results of the molecular tests. Our main objective was indeed to correlate the results of molecular testing to the diagnosis of congenital toxoplasmosis in a dynamic view which would be useful to the practitioner.

**Practical interest of the predictive values of molecular diagnosis of congenital toxoplasmosis in the prenatal period using amniotic fluid and at delivery using placenta and cord blood.**
The calculated pre- and post-test risk curves (Figure 2) we used here allow considering the results of molecular diagnosis of congenital toxoplasmosis in a novel manner; they should be helpful to obstetricians for day-to-day council about the risk of congenital toxoplasmosis to women who seroconverted during pregnancy. Indeed, to our knowledge, council is generally given according (i) to congenital toxoplasmosis prevalence or (ii) to a positive PCR result on amniotic fluid and/or on cord blood. A negative PND is considered of little value due to the presence of false negatives. Here, we show the respective weight of each molecular test result according to the gestational age at maternal infection whether they are positive or negative. This gives access to a larger amount of information and, specifically, gives the actual risk of congenital toxoplasmosis in case of a positive (PPV curve) or a negative (1-NPV curve) PCR result at a given time point, this being for the three types of samples used. Thus, if a positive PCR result using amniotic fluid and/or cord blood obviously affirms the diagnosis of congenital toxoplasmosis (23), the presence of a negative result using amniotic fluid considerably lowers the probability that transmission of the parasite occurred, particularly after 20 WA. Thus, a negative PCR result using amniotic fluid appears more helpful than generally thought, especially for mid-term and late maternal infections.

Due to the high number of false negative results, which makes the (1-NPV) curve overlap the prevalence curve, a negative cord blood PCR result is not very informative at any stage of the pregnancy. Using placenta, in spite of a moderate sensitivity and specificity, both positive and negative PCR results appear highly informative due to the large distance between the negative and, particularly positive, post-test risk curves and the prevalence curve (Fig. 2C).

It should be stressed that the curves presented here may be used by any group as long as the performances of the molecular diagnosis are similar to ours; without the amount of clinical data our laboratory has, this technically requires a PCR efficiency as close to 2 as possible.
and an analytical sensitivity ≤ 0.5 tachyzoite per reaction (11, 36); otherwise, the curves would have to be built by the users.

Performances of the parasitological diagnosis

In this study, the 'gold standard' for the definite diagnosis of congenital toxoplasmosis was the reference definition of criteria created by the European research Network on Congenital Toxoplasmosis (23), and all definite diagnoses of congenital toxoplasmosis were asserted by serologic follow-up after birth; thus any falsely negative or positive molecular diagnosis could be identified. The sensitivity at 86% reported here for our PCR assay is among the highest ever published for this diagnosis for large cohorts, which varies from 65% to 92% (reviewed in (3), and (5, 33, 38, 40, 43)). It is noteworthy that, in our study, the seven cases of congenital toxoplasmosis that were not detected in utero were subclinical forms; all symptomatic cases (n = 17) were diagnosed during the prenatal period by PCR. Moreover, no false positive results were observed, yielding 100% specificity. The specificity reported in the literature varies from 94-100% (reviewed in (3), and (5, 34, 38, 40, 43)). An absolute specificity is compulsory in the context of PND, as false positives may generate (i) at worse, an unwarranted decision of termination of pregnancy and (ii) unnecessary treatment, at least until delivery (and, in some cases, for the first year of age).

Placenta testing by PCR and/or mouse inoculation generally yields low sensitivities (under 60%) (5, 6, 10, 17, 18, 24, 29). Recent reports yielded conflicting results (16, 29), rendering the clinical relevance and usefulness of placenta testing for this diagnosis controversial; these two reports essentially differed by the sensitivity of the PCR assay used: 71% (20/28) versus 25% (13/51), respectively. In our experience, the search for Toxoplasma in placenta by PCR was highly sensitive (79% (31/39)); but, as also shown by others (reviewed in (3) and in (31)), a positive PCR using placenta was not always correlated with congenital toxoplasmosis, here in 16 cases, yielding a relatively high rate of false positive results (6 %). Yet, three of these
were also mouse inoculation-positive, indicating that these false positives were most likely due to a colonization of the placenta without transmission of the parasite to the fetus, this being better detected using a highly sensitive PCR assay. A positive placenta may be regarded as a clue for the diagnosis of congenital toxoplasmosis that needs to be confirmed by a positive cord blood and/or a positive serologic response in the newborn/infant.

With respect to cord blood, this is, to our knowledge, the first report of sensitivity and specificity of both PCR and mouse inoculation using this sample; only one study using mouse inoculation reported a sensitivity at 16% (4/25 cases) (24). Here, in two cases where molecular PND and serologic screening at birth were negative, the first argument to affirm the diagnosis of congenital toxoplasmosis was a PCR-positive cord blood; these results allowed starting drug treatment immediately after birth. Thus, we consider that, in spite of its low sensitivity, and due to its high specificity and PPV, PCR testing of cord blood remains an important step in the diagnosis of congenital toxoplasmosis, this is even more important when PND was negative and when serology at birth is not contributive. It should also be noted that, in our experience, a positive cord blood was always associated with a positive placenta.

Mouse inoculation, the conventional reference method, has proved consistently less sensitive than PCR in the context of PND (reviewed in (3) and (5, 21, 24, 30, 33, 40)), which was confirmed in this study. Nevertheless, there is a general consensus in France to continue including this method in routine diagnosis, due to a few reports from some groups of congenital toxoplasmosis cases with a positive mouse inoculation and a negative PCR on amniotic fluid (5, 21, 33). Moreover, mouse inoculation is the only method to isolate strains for genotyping, evaluation of pathogenicity or drug sensitivity, etc. (1): in our hands, as compared to amniotic fluid and cord blood, the placenta is the best suited sample material for this. Our figures for the sensitivity of mouse inoculation are different from previous reports: thus, the 38% and 71% we report here for mouse inoculation of amniotic fluid and placenta,
respectively, have to be compared with 71% and 52% reported by Bessieres et al. (5). Two differences with the protocol used by this center might explain these discrepancies: they use a higher inoculum of amniotic fluid and immunofluorescence instead of Toxoscreen DA for mouse serologic screening.

**Serologic screening at birth and during post-natal follow-up.**

A serologic follow-up of the child using a combination of ‘state of the art’ methods is the only way to assert a definite diagnosis of congenital toxoplasmosis and to rule out a falsely negative or positive molecular diagnosis. The 62.2% and 59.5% sensitivity of neonate IgM and IgA detection reported here are similar to the 64-70% for IgM, and the 53-65% for IgA reported in most series with cohorts ranging from 27 to 233 congenital toxoplasmosis cases (5, 6, 24, 30, 42). Testing both isotypes increased sensitivity to 68%, which was also observed by Wallon et al. 1999 (IgM 67%, IgA 54%, IgM and/or IgA 73%), Naessens et al. 1999 (IgM 41%, IgA 64%, IgM and/or IgA 66%) Bessières et al. 2001 (IgM 50%, IgA 60%, IgM and/or IgA 81%) and Bessières et al. 2009 (IgM 64%, IgA 53%, IgM and/or IgA 73%).

With respect to follow-up, we report a low percentage of loss during follow-up (13%) and a long follow-up period (1 year). In our cohort, the post-natal serologic follow-up did not detect any further case of congenital toxoplasmosis than those detected by molecular methods and neonatal serology. Nevertheless, in seven cases, the confirmation of a positive molecular PND was not obtained at birth but only during long-term follow-up.

**In total**, our experience confirms the usefulness and accuracy of a highly performing molecular diagnosis to detect congenital toxoplasmosis, both prenatally and at birth. The novelty of our study relies upon the consideration of the curves of pre- and post-test risks obtained from this molecular diagnosis for estimating the actual risk of congenital toxoplasmosis throughout the course of pregnancy. We therefore obtain a dynamic interpretation according to gestational age at maternal infection that is highly informative; this
should help microbiologists, obstetricians and pediatricians to better assess the actual risk of congenital toxoplasmosis after a correct molecular test has been performed, and thus guide the decision to treatment.

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References


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<td>91 [59-100]</td>
<td>89 [83.8-98.1]</td>
<td>0.21 [0.09-0.31]</td>
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<td>50 [59-100]</td>
<td>100 [15.8-100]</td>
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<td>100 [98-100]</td>
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</table>

WA: weeks of amenorrhea, b: values expressed as percentages with 95% confidence limits between brackets, c: nc: not computable, d: The [0-6 WA] and [7-15 WA] periods were distinguished due to a significantly different transmission rate between them (see Supplemental Material Table S2); however, the performances of the biological tests showed no statistical differences, hence we fused the calculations for both periods.
Figure legends

Figure 1. Description and outcome of the cases of *Toxoplasma gondii* maternal infection analyzed in this study.
Flow diagram. Top boxes: samples and biological tests used in this study. Below: description of cases. PND: prenatal diagnosis; AF: amniotic fluid.

Figure 2. Assessing the actual risk of congenital toxoplasmosis using post-test predictive values of amniotic fluid, placenta and/or cord blood PCR results.
Determination of the final diagnosis at the end of child follow-up allowed calculating, in our cohort, the prevalence of congenital toxoplasmosis (or pre-test risk) according to gestational age at maternal infection, all along the course of pregnancy (bold curve). We then calculated the same risk but differentiating, for each case, positive and negative PCR results (post-test probabilities of being infected). Closed triangles: post-test risk curves when the PCR test was positive (PPV); closed lozenges: post-test risk curves when the PCR test was negative (1 - NPV). These curves are represented on three graphs for amniotic fluid (A), placenta (B) and cord blood (C). All the cases of this study were reported on the logistic regression curves.