Maternal anti-*Toxoplasma* treatment during pregnancy is associated with reduced sensitivity of diagnostic tests for congenital infection in the neonate

Running Title: Anti-toxoplasma treatment impacts neonatal diagnosis

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Key-words

Congenital toxoplasmosis, Toxoplasma gondii, serology, IgM, IgA, western-blot, maternal treatment, neonatal diagnosis, qPCR

Abstract

Neonatal diagnosis of congenital toxoplasmosis is based on the combination of serological and molecular tests. Maternal screening and treatment differ according to national policies, and may impact on the sensitivity of diagnostic methods in infants at birth.

In this multicenter study, 115 neonates born to 61 treated (53%) and 54 (47%) untreated women were retrospectively included in three centers (France, Serbia, USA) to assess the impact of maternal anti-*Toxoplasma* treatment on the performance of neonatal workup at birth (neosynthesized anti-T*oxoplasma* IgM, IgA and IgG, and qPCR), using univariate and multivariate approaches.

Independently of the time of maternal seroconversion, the serological techniques were differently impacted by maternal treatment. The detection of IgM by ISAGA and western-blot (WB) dropped from 90.7% and 88.2% in untreated neonates to 53.3% and 51.9% in treated neonates ($p<0.05$), whereas IgM ELISA and IgA ISAGA were not significantly affected by maternal treatment. A two-fold reduction in the sensitivity of neosynthesized IgG by WB was also observed in case of treatment during pregnancy (37.7% versus 82.3%). Interestingly, the effect of treatment was shown to be duration-dependent, especially for IgM detection, when treatment course exceeded 8 weeks, whatever the therapy. The sensitivity of *Toxoplasma* PCR in blood was also lowered by maternal treatment from 39.1% to 23.2%.

These results highlight that anti-*Toxoplasma* therapy during pregnancy may set back biological evidences of neonatal infection at birth, and underline the need for a careful serological follow-up of infants with normal workup.

INTRODUCTION

Toxoplasmosis is a widespread protozoan foodborne infection (1, 2), affecting about one third of humans, with great differences in prevalence according to geographical areas (3). Hence, public health policies vary among countries, as the disease burden is diversely appreciated (4, 5). Indeed, *Toxoplasma gondii* infection is largely asymptomatic, except in immunocompromised subjects. It can also be responsible for congenital infection when primary infection is acquired during pregnancy. The only way to know if a pregnant woman has acquired toxoplasmosis during pregnancy is to determine her serological status at the beginning of gestation, and repeat serological testing regularly until delivery, if it shows an absence of protective immunity. Such serological screening has been implemented in some countries including France, Austria, Belgium, Italy (6–8), to allow early maternal anti-*Toxoplasma* therapy, and reduce vertical transmission and severe fetal damages (9–15). The usual treatment consists in spiramycin (SPI) administration until delivery (16), but if prenatal diagnosis demonstrates the presence of *Toxoplasma* DNA in amniotic fluid, it is strongly recommended to switch SPI to the association of pyrimethamine and sulfadiazine (PYR-SD), as the latter is more potent to reduce fetal sequelae (17). However, in about 10% of cases, neonates are diagnosed after birth, despite a negative prenatal diagnosis, and in about 30% of cases, prenatal diagnosis is not performed because of late infection during gestation (18). Therefore, postnatal diagnosis is essential to diagnose infected neonates and start treatment, as recommended (19–21).

Clinical and biological work-up for postnatal diagnosis of congenital toxoplasmosis relies on a combination of methods including, parasite detection in placenta or amniotic fluid collected during delivery (18), cord blood or newborn blood, by PCR and serological screening (22, 23). The serological workup is now well-established (6, 21, 24) and usually relies on the detection of specific IgA or IgM in the neonate serum. As specific IgG antibodies developed by the mother are transferred to the fetal

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compartment, the only way to detect specific IgG synthesized by the neonate himself, is to characterize anti-*Toxoplasma* IgG profiles of mother and newborn paired serum samples at birth by western-blotting. This technique has been added to the routine serological workup since many years in most reference labs in France (25, 26), and is widely used across Europe. Sensitivity data of these techniques have been published over years, but show various performances according to studies and countries.

A possible impact of maternal treatment has been suggested for parasite detection in placenta or IgM detection in neonates, with a higher rate of positive qPCR or positive IgM, respectively, when mothers had received SPI, compared to PYR-SD (18, 27). However, both studies were conducted in France, where women usually receive specific therapy during pregnancy, thus the comparison of the sensitivity of biological tests in neonates born to treated and untreated mothers was not addressed. Recently, Olariu et al. (28) reported a possible impact of maternal treatment on IgA and IgM detection, but the number of treated cases was small, and the trimester of infection, which was shown to influence IgA and IgM antibody detection in the neonate, was not taken into account.

Therefore, the aim of the present study was to assess the impact of maternal anti-*Toxoplasma* treatment during pregnancy on the sensitivity of various tests in neonates at birth, using a multivariate analysis performed on data from three countries with different national policies, i.e. maternal screening and treatment (France), occasional maternal screening (Serbia), and no maternal screening (USA).

MATERIALS AND METHODS

Ethics

All analysis were performed during routine work-up, as implemented in the three participating centers. Data were recorded anonymously. The study design was approved by the local ethics committee of the University Hospital of Rennes (approval number: #20.08).

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Patients

All congenitally infected infants diagnosed over a 10-year period (2010-2019) in the three labs (Dr. Jack S. Remington Laboratory for Specialty Diagnostics, Palo Alto, CA, USA, (JSRLSD), National Reference Laboratory for toxoplasmosis from Serbia (CNLTS) and Rennes University Hospital Parasitology lab (RUH-PL)) were retrospectively included if a blood sample had been analyzed at birth or during the first month of life. Diagnosis of congenital toxoplasmosis relied on a positive prenatal diagnosis (parasite DNA detection by qPCR on amniotic fluid), and/or detection of specific IgM or IgA in peripheral blood at >7 days of life, or positive *Toxoplasma* qPCR in peripheral blood or cerebrospinal fluid, or detection of neosynthesized IgG or IgM by western-blot.

Other relevant data were recorded for analysis: age of gestation at the time of maternal infection, type, date of onset and duration of anti-*Toxoplasma* targeted therapy, gender of the newborn, clinical signs at birth or during the first year of life. When the date of maternal infection could not be known with accuracy, due to the absence of early serological screening, the estimation was graduated as "1^{st} , 2^{nd} or 3^{rd} trimester", according to clinical or imaging findings or serological results during pregnancy or "undetermined" when discovered only after birth.

Serological methods

Anti-*Toxoplasma* IgM were detected by ELISA (Platelia Toxo IgM, Biorad, Marnes-la-Coquette, France) and ISAGA (BioMérieux, Marcy-l'Etoile, France) in CNLTS and RUH-PL, and only by ISAGA in JSRLSD. Anti-*Toxoplasma* IgA were detected by ISAGA IgA (BioMérieux) in CNLTS and RUH-PL, and by an in-house ELISA in JSRLSD. Comparison of maternal and neonatal IgG and IgM antibody profiles was performed using the *Toxoplasma* WB IgG/IgM assay (LDBIO, Lyon, France) in CNLTS and RUH-PL.

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ELISA indexes for IgM and IgA were positive when ≥1 and negative when <0.8. For performance calculation, ELISA indexes in grey zone were merged with positive results. ISAGA IgM and IgA scores were positive when ≥3, according to the manufacturer's recommendations. When a positive result was obtained on a cord blood sample using a quantitative test, it was taken into account only if it could be confirmed on peripheral blood or if WB could rule out contamination of cord blood by maternal IgM (different profiles).

WB profiles of the mother and her baby were compared to determine if additional bands were observed in the neonatal serum (neosynthesis by the infected neonate). When cord blood samples were tested, if the IgM patterns of the mother and baby were similar, then the cord blood was considered contaminated by the maternal serum and was excluded from the study. When maternal and neonatal patterns differed by only one faint band, the WB was considered as doubtful. In both instances, if there were no other arguments in favor of the diagnosis of infection, all tests were repeated on a neonatal blood sample collected several days later.

Toxoplasma real-time PCR

For parasite DNA detection, 200 µL of total blood or cerebrospinal fluid (CSF) were extracted using Qiamp DNA Mini-kit (Qiagen) and 5 µL of DNA was used for amplification. In CNLTS and RUH-PL, the *Toxoplasma*-specific quantitative real-time PCR targeted the repetitive *rep529* sequence and was carried out as previously described (18, 29, 30). In JSRLSD, the PCR used two target sequences from the B1 gene (22).

Statistical analysis

All serological and PCR results were included as dichotomous variables (positive / negative), with borderline test results classified as positive, and were analyzed according to maternal treatment.

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An univariate analysis was performed on population characteristics according to treatment group. The association of the different test results with qualitative variables (gender, type of sample, trimester of pregnancy, treatment group) was analyzed using Pearson's $\chi 2$ test or Fisher's exact test. For continuous variables (age at time of sampling and duration of treatment) for which the Skewness and Kurtosis tests showed non-Gaussian distribution, we used the nonparametric Mann–Whitney U test, and the distributions were displayed as medians with interquartile ranges [25th;75th percentile].

Multivariate analysis was then carried out, and a logistic regression model was set up with each particular test result as an outcome variable and the trimester of infection, the gender, the newborn age at sampling (in days), the center, and the maternal treatment (yes / no) as covariates. The duration of treatment (by categories ≤8 / >8 weeks or in number of weeks) and the type of treatment (SPI or SPI ± PYR-SD) were further analyzed on the treated group. Only variables that were found to be significant by univariate analysis, with $P < 0.2$, were tested forward by stepwise Wald logistic regression. The Odds ratio ([OR]) and 95% confidence intervals (95% CI) were used to describe the associated factors if $P < 0.05$.

All statistical tests and procedures were performed using the IBM SPSS 21 statistical package (IBM, NY, USA).

RESULTS

Patient file

A total of 115 mother-neonate pairs were included from the 3 centers; 46 from France, 26 from Serbia and 43 from the USA. Congenital infection was diagnosed antenatally (n=27) and/or after birth on the basis of neosynthesized antibody detection or positive PCR on newborn samples (Supplementary Table 1). The first positive post-natal test was obtained as soon as birth until one year of age when treatment

was stopped (Supplementary Table 1). Neonates were aged from 0 to 35 days (median days 3 [1; 11], when the first blood sample was obtained and included in the study. The time of sampling was earlier in newborns from treated mothers than from untreated ones, as a result of the follow-up protocol implemented in case of maternal *Toxoplasma* infection in Europe (Table 1). Blood samples included 46 cord blood and 69 peripheral blood samples. Most samples were taken before 5 days of life (n=70) (Table 1). The frequency of detection of specific IgG, IgM, or IgA neosynthesized antibodies did not depend on the sample type in any of the analyzed diagnostic tests ($p>0.05$). Maternal seroconversion occurred during the first (T1), second (T2) and third (T3) trimester in 14 (12.2%), 28 (24.3%) and 36 (31.3%) of cases, respectively, while the date of maternal infection remained unknown in 37 (32.2%), of which 34 were symptomatic cases (Table 1).

An anti-*Toxoplasma*-targeted therapy during pregnancy was given to 61 (53.0%) pregnant women. The proportion of treated women did not differ according to the trimester of seroconversion (Table 1). As expected, the median duration of treatment was significantly shorter when infection was diagnosed in T3 (median weeks 5 [2; 7]) in comparison with T2 (median weeks 12 [7.25; 14]) and T1 (median weeks 17.5 [8; 24], $p<0.001$), respectively. The treatment consisted of SPI alone (n=34), PYR-SD (n=8) or SPI followed by PYR-SD (n=19). Interestingly, the proportion of infections acquired during the first, second and third trimester didn't differ among treated and untreated mothers (Table 1), ruling out a possible interplay of these two parameters in the interpretation of results.

The incidence of *Toxoplasma*-related clinical manifestations within the first year of follow-up reached 53.1% (60/113) (Table 1). The main disorders included cerebral lesions, i.e intracranial calcifications, ventriculomegaly and meningitis in 47/60 (78.3%), ocular lesions in 26/60 (43.3%), and growth retardation/prematurity in 7/60 (11.7%) neonates. The rate of newborns with clinical signs was significantly lower when treatment was given during pregnancy (31.1% versus 75.9% in treated and untreated groups, respectively) (Table 1).

Maternal treatment is associated with lower frequency of detection of anti-*Toxoplasma* IgM and IgG in the neonate, but not of IgA

As a first approach to investigate the impact of maternal anti-*Toxoplasma* treatment on the results of diagnostic methods, we calculated the overall sensitivity of each test for prenatally treated and untreated infants. The overall sensitivity of IgM detection by ELISA and/or ISAGA was lower in infants born to treated mothers ($p<0.0001$, Table 2). However, when analyzing separately each technique, the ELISA-based IgM detection was not affected by treatment, whereas the sensitivity of IgM ISAGA dropped from 90.7% to 53.3%, when treatment was administered ($p<0.0001$, Table 2). WB-based IgM detection was also significantly lower in the treated group, as it was positive in only 55.8%, compared to 88.2% without treatment ($p<0.05$). This reduction was also observed when gathering doubtful IgM WB profiles (only one additional faint band obtained with the neonate's profile, compared to the mother's IgM profile) with negative results instead of with the positive ones ($p=0.009$, data not shown). An above two-fold reduction of neosynthesized IgG detection by WB was also observed in neonates from treated mothers, 82.3% versus 37.7% ($p<0.01$). No significant differences were observed in the detection of specific IgA (ELISA or ISAGA) according to maternal treatment (Table 2).

To further analyze the differences observed between ISAGA and ELISA for IgM detection, we evaluated the sensitivity on serum samples analyzed concomitantly with both techniques (Serbian and French neonates). Results showed that poorer ISAGA sensitivity was associated with maternal treatment, whereas ELISA sensitivity was even in both treated and untreated groups (Table 3).

As the absence of specific IgM detection in the neonate could be solely due the physiologic disappearance of antibodies before birth when infection occurred in early pregnancy, we then performed a multivariate analysis to confirm the results obtained by univariate analysis. Interestingly, results showed that treatment significantly altered IgM detection by ISAGA and WB ($p<0.001$ and

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208 p<0.01, respectively), as well as neosynthetized IgG detection by WB (Table 4), thus confirming
209 univariate analysis. Multivariate analysis also showed that the trimester of maternal infection influenced
210 the positivity of these serological tests with more frequent positivity for infections acquired during the
211 third trimester, while the age of the newborn at the time of sampling had no effect (Table 4 and
212 Supplementary Table 2). Unexpectedly, the gender was significantly associated with IgM detection, with
213 male and female infants being most likely to have positive ISAGA IgM, and positive WB IgM, respectively
214 (Table 4 and Supplementary Table 2).
215
216 **The duration of anti-*Toxoplasma* therapy during pregnancy is unequally associated with reduced**
217 **sensitivity of serological tests**
218 To further analyze if the duration of maternal anti-*Toxoplasma* therapy influenced the sensitivity of
219 diagnostic methods, we divided the group of 61 infants from treated mothers into two sub-groups,
220 according to the length of maternal treatment, i.e. ≤8 weeks or >8 weeks of any treatment.
221 Interestingly, the duration of treatment interfered heterogeneously with the detection of IgM, IgA or
222 IgG. The detection of specific IgA, regardless of the method, was not altered, but when distinguishing
223 ISAGA and ELISA, it appeared that ISAGA sensitivity was significantly reduced if treatment course was >8
224 weeks (p <0.05, Table 5). Sensitivity of IgM detection, whatever the technique used (ELISA, ISAGA or
225 WB), was dramatically decreased when treatment exceeded 8 weeks (p<0.001, p<0.01, p=0.001,
226 respectively) (Table 5). Overall, the range of IgM detection decreased from 72-79% to 29-32%, when
227 treatment lasted ≤8 weeks and >8 weeks, respectively, depending on the method. By contrast, IgG
228 detection by WB was not significantly modified according to the cut-off of 8 weeks of treatment (Table
229 5). The duration of treatment seems to have a progressive effect, as illustrated in Fig 1.
230 Multivariate analysis using the same cut-off of treatment duration (≤8 weeks or >8 weeks), confirmed
231 these findings (Table 5). Additionally, when taking into account the precise duration of treatment

(number of weeks), a longer duration of treatment was associated with a lower sensitivity of ISAGA IgA, as well as of IgM detection by any technique, but no impact on neosynthesized IgG detection by WB (Table 6). Overall, among the 61 treated neonates, no statistically significant difference ($p > 0.05$) was found between neonates treated with SPI or PIR-SD ± SPI, in any serological test results (Table 6).

Prenatal therapy is associated with decreased sensitivity of the detection of parasite DNA in the neonate at birth

Eighty-four neonate specimens consisting of blood (n=66) or CSF samples (n=18) were also collected for *Toxoplasma* detection by qPCR. The sensitivity dropped from 43.6% to 24.4% in neonates from untreated and treated mothers, respectively (Table 2). When the precise duration of treatment was analyzed for each type of treatment, regression analysis showed that there was no effect of SPI alone, whereas there was a significant time-dependent effect of PYR-SD on the DNA detection by PCR (Table 6).

DISCUSSION

The laboratory diagnosis of congenital toxoplasmosis at birth requires the use of multiple techniques to prove infection of the neonate, through detecting either antibody neosynthesis by the newborn, or circulating parasite DNA. Postnatal screening of neonates is paramount in three situations: i) when evocative clinical signs are present at birth and the mother has not benefited from *Toxoplasma* serological screening during gestation, ii) when the mother acquired toxoplasmosis during gestation and the prenatal diagnosis was negative, and iii) when the mother acquired toxoplasmosis, but prenatal diagnosis was not performed (late infection during pregnancy).

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As most French women with primary infection during pregnancy are given specific treatment, we included data of infants from Serbia, where maternal screening has been only recently adopted, and from the USA, where women are diagnosed and treated only in case of ultrasound anomalies. We chose to include only early samples, because we wanted to draw conclusions on the effect of maternal on the early diagnosis of congenital toxoplasmosis. To ensure perfect interpretation of IgM or IgA detection in cord blood samples, we considered them as positive only if IgM neosynthesis by the newborn was confirmed by WB, thus ruling out maternal contamination for both IgM and IgA.

Using univariate and multivariate analyses, we found strong evidence that maternal treatment was associated with a dramatic decrease in the sensitivity of IgM and neosynthesized IgG in the neonate. Whereas a direct impact of treatment on parasite replication and dissemination was expected, as it is assumed to reduce parasite loads (27), the demonstration of an impact on antibody synthesis by the newborn is a novelty. Indeed, it could be hypothesized that the absence of IgM or IgA detection in newborns was due to a transient synthesis by the fetus, particularly if infection occurred early in gestation. Indeed, we observed that the positivity of tests was associated with the trimester of infection (Table 4), with a higher detection rate for infections acquired during the third trimester (Suppl. Table 2), which also coincides with shorter courses of treatment. However, there was no differences in the time of infection among treated and untreated mothers (Table 1), thus the time of infection cannot bias the conclusions on the effect of treatment. Taken together, multivariate analysis clearly demonstrated an association of treatment with a lower sensitivity of IgM and neosynthesized IgG antibody detection that could be explained by a delayed synthesis. Additionally, this effect was time-dependent, with an apparent cut-off of 8 weeks of any treatment (SPI or PYR-SD) for most serological tests. Besides, the ISAGA technique was more impacted by maternal treatment than the ELISA technique. Since the IgM ISAGA technique uses “global” antigens from entire parasites, this finding would suggest that maternal treatment could more likely affect the ability of the fetus to react against surface antigens, than against

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278 cytoplasmic antigens. Why SPI and PYR-SD administered to the mother had roughly the same effect on
279 the fetus or neonate antibody response is difficult to explain, as SPI is known as hardly transferring
280 through the placental barrier. A previous study by Gilbert et al. found no impact of maternal treatment
281 on the sensitivity of IgM detection by multivariate analysis, but the group treated with PYR-SD was
282 compared to a control group including both SPI-treated and untreated mothers (31).
283 In contrast to our results, Naessens et al. investigated IgM detection in peripheral blood samples from
284 86 congenitally infected newborns and found no significant effect of treatment despite an apparent
285 decrease of sensitivity (85% in untreated versus 25% in treated, p=0.19) ; they attributed it to the
286 gestational age of maternal infection rather than to prenatal treatment (32). However, the vast majority
287 of mothers (75%) had been treated, and the duration of treatment was not known with accuracy, nor
288 the time of maternal infection, as monthly serological screening was not current practice in most
289 centers. In a Brazilian study primarily aiming at measuring the duration of IgM synthesis by infected
290 infants, Lago et al. observed that in 23/28 neonates whose mother had been diagnosed with
291 seroconversion during pregnancy, IgM were detected during the first month of life. However, the
292 difference in sensitivity between treated (75%) and untreated (88%) groups was not statistically
293 significant (p=0.4) (33).
294 While IgA detection appeared not to be affected by treatment using univariate analysis, there was a
295 trend towards a time-dependent effect of treatment in infants from treated mothers, irrespective of the
296 type of treatment. Again, this was significant only with the ISAGA technique, but it must be
297 acknowledged that there was a small number of samples analyzed with ELISA. Interestingly, it has been
298 shown in experimental murine infection that specific IgA synthesis was the least affected of all antibody
299 classes by several anti-*Toxoplasma* chemotherapeutic regimens (34).
300 In contrast, despite a significant effect of treatment on the detection of neosynthesized IgG by WB, it
301 was not related to the duration of maternal treatment. It was not related to the time of sampling, i.e.

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302 the age of the newborn, either (Table 4). In infants from untreated mothers, neosynthesized IgG were
303 found in 4/5 (80%) in the early days after birth (0-4 days) and in 7/9 (78%) from day 15 to 35 (data not
304 shown). Few reports are available on the detection of neosynthesized IgG using WB. In a cohort of 55
305 neonates, a French multicenter study reported a sensitivity of IgG WB similar to ours at birth (37.7% in
306 the treated group), increasing to 40% during the first 10 days of life, and to 63.3% between 0.5 and 1.5
307 months (35). Similar findings were published in Brazil, where the IgG WB was positive in 40% (6/15) of
308 infected newborns born to treated mothers up to 3 months of age (36). In another Brazilian series of
309 newborns aged up to 1 month and born to women who had not been given treatment during
310 pregnancy, the sensitivity of IgG WB reached 73.5 % (130/177), which is not far from the results
311 obtained in our untreated group (82.3%) (37). However, they found a lower sensitivity of IgM WB (54.8
312 %) compared to that of IgG WB.
313 In a previous study, Olariu et al (2019) analyzed IgM and IgA detection in infants from 25 treated and
314 164 untreated mothers, and similarly found that IgM were less frequently detected in infants of treated
315 mothers (44%) than of untreated mothers (86.6%, $p<0.001$)(28). The detection of IgA was not modified
316 according to maternal treatment, either ($p=0.06$). However, they did not take into account the
317 gestational age at the time of maternal infection, which is a major factor impacting the sensitivity of
318 serological tests (27, 32). In addition, samples were obtained within the first six months of life, whereas
319 in the present study, we focused on the results obtained early after birth (mean age of 7 days).
320 It is interesting to note that, in case of maternal anti-*Toxoplasma* treatment, the detection of IgA was
321 the most sensitive approach to detect neonatal infection, outperforming IgM and IgG-based screening
322 (68.4% versus 59% and 38%, respectively), while it was less sensitive in untreated cases.
323 Still, the overall sensitivity of IgA and IgM detection (71.8% and 73.9%, respectively) is quite high
324 compared to previous reports, which showed sensitivity ranges from 47.8 to 72.5% and 44 to 67.5%, for
325 IgA and IgM, respectively, irrespective of the technique (24, 27, 31, 35).

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Whereas the contribution of PCR testing in AF has been extensively evidenced for prenatal diagnosis, the use of PCR on newborn blood or CSF at birth has been sparsely investigated (22). In our cohort, the sensitivity of blood PCR was low (28.6%) but it is interesting to note that it ranged from 23% to 39% in treated and untreated infants, respectively. Unexpectedly, while univariate analysis was significant, multivariate analysis showed that treatment onset did not impact PCR positivity. However, among treated neonates, the longer the treatment, the lower the sensitivity of PCR, provided that the treatment was PYR-SD, which is in agreement with the expected in utero anti-parasitic effect of this bi-therapy. The trimester of pregnancy had no impact on the positivity of PCR, while it might be expected to find more positive PCR results when infection occurred at the end of pregnancy, due to a shorter course of treatment. These findings may suffer from a bias of center, as the proportion of positive cases was higher in the JSRLSD (38% versus 23% in RUHPL), but 37 cases were excluded from the multivariate analysis because of missing data, thus the power of the analysis may have been impaired.

The strength of our study is that it was performed on a well-balanced cohort of prenatally-treated and untreated infants, diagnosed prospectively in three reference centers. A limitation can result from the uncertainty of the time of maternal infection in some US cases, as no serological follow-up was undertaken, which led us to discard 32% of cases for the multivariate analysis.

Taken together, we provide evidence that anti-*Toxoplasma* therapy in the mother may contribute to set back serological confirmation in the neonate, and underlines the need for careful serological follow-up of neonates even if the workup at birth is normal. We also recall that prenatal diagnosis can detect at most 90% of infected fetus, thus postnatal serological follow-up is essential.

ACKNOWLEDGMENTS

This study was supported by the Ministry of Education, Science and Technological Development of Serbia, through project no. III 41019; and by a grant to the Institute for Medical Research (contract no.

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350 451-03-68/2020-14/200015). The authors also received a grant from Campus France (PHC Pavle Savic
351 no.40734WC) (Serbian project number 451-03-01963/2017-09/15).

352

353 **LEGENDS TO FIGURES**

354 **Fig. 1: Sensitivity of diagnostic tests according to the duration of treatment.**

355 Statistical significance was graduated as * ($p<0.05$) and ** ($p<0.01$). Data obtained for ELISA IgA are not
356 shown because only 6 infants had been treated in utero.

357

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468 **Table 1. Population characteristics (N=115) and comparison according to treatment group**

469

Characteristics	Maternal treatment during pregnancy		Univariate analysis [$]
	No (N = 54)	Yes (N = 61)	p
Sex ratio (M/F)	0.69 (22/32)	1.10 (32/29)	0.209, ns
Type of blood sample, n (%)			<0.0001
Cord blood (n=46)	4 (7.4)	42 (68.8)	
Peripheral blood (n=69)	50 (92.6)	19 (31.1)	
Time of blood sampling, median (interquartile range)	10 (4-17)	1 (0-3)	<0.0001
0-4 days of life (n=70), n (%)	16 (29.6)	54 (88.6)	
5-14 days of life (n=21), n (%)	20 (37.0)	1 (1.6)	
15-35 days of life (n=24), n (%)	18 (33.3)	6 (9.8)	
Trimester of maternal infection, n (%)			0.063, ns
TI (n=14)	4 (7.4)	10 (16.4)	
TII (n=28)	2 (3.7)	26 (42.6)	
TIII (n=36)	11 (20.4)	25 (41.0)	
ND (n=37)	37 (68.5)	0	
Clinical manifestation during the first year of life, n (%)			<0.0001
Yes (n=60)	41 (75.9)	19 (31.1)	
No (n=53)	11 (20.4)	42 (68.9)	
ND (n=2)	2 (3.7)	0	
Type of treatment, n (%)	na		na
SPI alone		34 (55.7)	
SPI then PYR-SD		19 (31.1)	
PYR-SD alone		8 (13.1)	

470 [$] Pearson's $\chi 2$ test or + Fisher's exact test compared distributions in treated and untreated groups

471 na: not applicable; nd: not determined; ns: not significant; SPI: spiramycin; PYR-SD: pyrimethamine-sulfadiazine

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474 **Table 2: Sensitivity of serological tests and qPCR in newborns according to treatment group (N=115)**

Diagnostic method	Overall sensitivity according to maternal treatment % (n/N)			Univariate analysis p (Fisher's test)
	All N=115	Untreated N=54	Treated N=61	
IgA[R] (ELISA or ISAGA)	71.8 (79/110)	75.5 (40/53)	68.4 (39/57)	0.416
IgA ELISA	72.1 (31/43)	73.0 (27/37)	66.7 (4/6)	1
IgA ISAGA	71.6 (48/67)	81.2 (13/16)	68.6 (35/51)	0.526
IgM[R] (ELISA or ISAGA)	73.9 (85/115)	90.7 (49/54)	59.0 (36/61)	0.0001
IgM ELISA	59.2 (42/71)	68.8 (11/16)	56.4 (31/55)	0.564
IgM ISAGA	71.1 (81/114)	90.7 (49/54)	53.3 (32/60)	<0.0001
IgM WB	63.8 (44/69)	88.2 (15/17)	55.8 (29/52)	0.0198
IgG WB	48.6 (34/70)	82.3 (14/17)	37.7 (20/53)	0.0018
qPCR	33.3 (28/84)	43.6 (17/39)	24.4 (11/45)	0.104
Blood	28.8 (19/66)	39.1 (9/23)	23.2 (10/43)	0.254
CSF	50.0 (9/18)	50.0 (8/16)	50.0 (1/2)	1

475 [R] IgM and IgA ELISA values in the grey zone were grouped with positive results

476 Na, not applicable

477

Table 3. Compared sensitivity of ISAGA and ELISA assays for IgM detection (N=70) according to treatment group

	Sensitivity according to maternal treatment % (n/N) [£]		p (Fisher test)
	Treated	Untreated	
IgM ELISA[¤]	55.6 (30/54)	68.8 (11/16)	0.400
IgM ISAGA	51.9 (28/54)	81.2 (13/16)	0.045

£This analysis included only neonates for whom both IgM ELISA and IgM ISAGA were performed on the same sample

¤ IgM ELISA values in the grey zone were grouped with positive results

491 **Table 4.** Multivariate analysis of the effect of treatment, trimester of maternal infection, and age at

492 blood sampling on the sensitivity of diagnostic tests

493

Outcome Variable	Covariates			
Diagnostic method	Treatment p OR (95% CI)	Trimester of maternal infection p OR (95% CI)	Gender male p OR (95% CI)	Newborn age p OR (95% CI)
IgM ISAGA (n=144)	0.000 0.117 (0.041-0.333)	0.001 3.417(1.648-7.085)	0.021 2.680 (1.160-6.192)	0.104
IgM WB (n=69)	0.002 0.049 (0.007-0.322)	0.000 6.623 (2.461-17.874)	0.003 0.174 (0.055-0.547)	0.292
IgG WB (n=70)	0.005 0.115 (0.025-0.518)	0.007 2.931 (1.348-6.372)	ND	0.242
qPCR* (n=80)	0.480 0.292 (0.068 0.990)	ND	ND	0.007 1.091(1.024-1.162)

494 *blood or CSF

495 ND; not determined because the p value in univariate analysis was <0.2 (results not shown)

496

Table 5. Sensitivity of serological tests and qPCR in newborns from treated mothers (N = 61), and comparison according to treatment duration ≤8 or >8 weeks

Diagnostic test	Sensitivity according to the duration of treatment % (n/N)		Univariate analysis[$]	Multivariate analysis [§]	
	≤ 8 wks N = 33	> 8 wks N = 28	p	p	OR [95% CI]
IgA (ELISA or ISAGA) (N=57)	78.1 (25/32)	56.0 (14/25)	0.091	0.079	na
IgA (ELISA) (N=6)	33.3 (1/3)	100 (3/3)	1	nd	na
IgA (ISAGA) (N=51)	82.8 (24/29)	50.0 (11/22)	0.017	0.009	0.161 [0.041-0.629]
IgM (ELISA or ISAGA) (N=61)	75.6 (25/33)	39.3 (11/28)	0.005	0.04	0.208 [0.070-0.619]
IgM (ELISA) (N=55)	76.7 (23/30)	32.0 (8/25)	0.001	0.048	0.288 [0.083-0.972]
IgM (ISAGA) (N=60)	71.9 (23/32)	32.1 (9/28)	0.004	0.035	0.117 [0.026-0.517]
IgM (WB) (N=52)	78.6 (22/28)	29.2 (7/24)	0.001	0.001	0.112 [0.032-0.396]
IgG (WB) (N=53)	46.4 (13/28)	28.0 (7/25)	0.256	nd	na
qPCR [#] (N=45)	30.8 (8/26)	15.8 (3/19)	0.309	nd	na

na: not applicable
nd: not determined because the p value in univariate analysis was <0.2
[$] Fischer's exact test
[§] multivariate analysis was done using the treatment duration as qualitative variable (>8 or ≤8 weeks); data for other covariates are not shown

506 **Table 6: Sensitivity of diagnostic tests according to treatment duration (weeks)**

Diagnostic test	Statistical significance of treatment duration in weeks on diagnostic tests*					
	Any treatment (N=61)		SPI (N = 34)		PYR-SD ± SPI (N =27)	
	p-value [§] (Mann–Whitney test)	Regression Analysis[#] P OR [95% CI]	p-value [§] (Mann–Whitney test)	Regression analysis p OR [95% CI]	p-value [§] *(Mann–Whitney test)*	Regression analysis p OR [95% CI]
IgA (ISAGA)	0.052	0.047 0.929 [0.864-0.999]	0.069	0.056	0.375	ND
IgM (ELISA)	0.000	0.001 0.518 [0.581-0.962]	0.052	0.052	0.346	ND
IgM (ISAGA)	0.001	0.003 0.815 [0.440-0.956]	0.030	0.040 0.859 [0.7430.993]	0.002	0.004 0.864 [0.778-0.953]
IgM (WB)	0.000	0.001 0.857 [0.761-0.965]	0.000	0.027 0.872 [0.758 -0.901]	0.000	0.023 0.845 [0.730-0.977]
IgG (WB)	0.149	0.155	0.435	ND	0.755	ND
qPCR	0.124	0.193	0.615	ND	0.065	0.042 0.102 [0.011-0.902]

507 *data for other covariates are not shown
508 [§] comparison of the mean durations of treatment in patients with negative and positive tests results
509 [#] calculated on N= 51, 55, 60, 52, 53 and 45, respectively for the various tests
510 ND: not determined because the p value in univariate analysis was <0.2
511 SPI: spiramycin; PYR-SD: pyrimethamine-sulfadiazine
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