SEROLOGICAL AND MOLECULAR DIAGNOSIS OF *TOXOPLASMA GONDII* INFECTION IN PREGNANT WOMEN IN ROMANIA

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ABSTRACT

Introduction: Early diagnosis of *Toxoplasma gondii* infection in pregnant women allows the assessment of the risk of transplacental transmission and prevents the occurrence of congenital toxoplasmosis (CT) by initiating timely therapy.

Objectives: The current study aimed to improve the prenatal diagnosis of maternal toxoplasmosis using a combination of serological and molecular methods and to estimate possible risk factors in acquiring *T. gondii* infection.

Methods: Blood samples from 170 pregnant women enrolled in this study between January 2009 and December 2013 were investigated for the presence of specific antibodies to *T. gondii* by enzyme-linked immunosorbent assay (ELISA). IgG avidity test and PCR analyses on DNA fragments from two genetic markers were performed in all pregnant women who needed confirmation of their *T. gondii* infection status.

Results: By immunological analyses, 38.24% of total participants were detected *T. gondii* seropositive IgG and 17.65% *T. gondii* seropositive IgM. Of the 30 women serologically suspected for a recent infection, only 13.3% had all indications (positive specific IgM and IgA antibodies, low IgG avidity and positive PCR) for *T. gondii* maternal infection acquired during pregnancy at risk for CT. A significant association of *T. gondii* infection with consumption of undercooked meat ($p<0.05$) was reported.

Conclusions: IgG avidity test combined with PCR analyses performed prior to the use of antiparasitic therapy is the optimal diagnostic formula in suspicion of recently acquired *T. gondii* maternal infection with positive specific IgM antibodies. An educational system and a systematic screening of pregnant women for *T. gondii* infection are essentially indicated.

Keywords: toxoplasmosis, *Toxoplasma gondii*, pregnancy, ELISA, IgG avidity, PCR, *B1* gene, *rep529,* Romania.

REZUMAT

Introducere: Diagnosticul precoce al infecției cu *Toxoplasma gondii* în cursul sarcinii permite evaluarea riscului de transmitere transplacentară și previne apariția toxoplasmozei congenitale (TC) prin inițierea în timp util a unei terapii.

Obiective: Studiul curent a vizat îmbunătățirea diagnosticului prenatal al toxoplasmozei materne, printr-o combinație de metode serologice și moleculare, și estimarea potențialilor factori de risc pentru dobândirea toxoplasmozei.

Metode: Probe de sânge de la 170 de gravide înscrise în acest studiu între ianuarie 2009 și decembrie 2013 au fost investigate pentru prezența anticorpilor anti-*T. gondii* prin metoda ELISA. Testul de aviditate IgG și analizele PCR pentru doi markeri genetici au fost efectuate la toate gravidele care au necesitat o confirmare a stării lor de infecție cu *T. gondii*.

Rezultate: Prin analize imunologice, 38,24% din numărul total de participante au fost detectate *T. gondii* IgG pozitive și 17,65% *T. gondii* IgM pozitive. Dintre cele 30 de femei suspectate serologic pentru o infecție recentă, numai 13,3% au prezentat toate indicațiile (anticorpi specifici IgM și IgA pozitivi, aviditate IgG scăzută și PCR pozitiv) pentru infecția maternă cu *T. gondii* dobândită în timpul sarcinii cu risc de TC. De asemenea, s-a semnalat o asociere semnificativă a toxoplasmozei cu consumul de carne nepreparată termic ($p < 0,05$).

Concluzii: Testul de aviditate IgG, combinat cu analizele PCR efectuate înainte de utilizarea terapiei antiparazitare, este formula optimă pentru diagnosticul suspiciunii de infecție maternă cu *T. gondii* dobândită recent, cu anticorpi IgM specifici pozitivi. Implementarea unui sistem educațional și o examinare sistematică a gravidelor pentru infecția cu *T. gondii* sunt indicate în mod esential.

Cuvinte-cheie: toxoplasmoza, *Toxoplasma gondii*, sarcina, ELISA, aviditate IgG, PCR, gena *B1*, *rep529,* România.

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INTRODUCTION

Toxoplasmosis is a zooanthroponosis induced by the ubiquitous protozoan parasite *Toxoplasma gondii* for which the definitive host is the cat. This obligate intra-cellular parasitic microorganism was estimated to infect up to one third of the world's population. The intermediate host may acquire this infection primarily by ingestion of raw or undercooked meat containing tissue cysts, or alternatively by water, food contaminated with oocysts excreted in the feces of infected cat [1-3]. Although immunocompetent subjects are usually asymptomatic or may be mild and self-limited with or without lymphadenopathy, *T. gondii* infection in immunodeficient patients and fetuses may be a significant cause of morbidity and even mortality. Congenital transmission can occur following primary maternal seroconversion and the presence of *T. gondii* circulating tachyzoites in the pregnant woman. The risk of vertical transmission varies with pregnancy status at the time of primary infection (10% -25% in the 1st trimester, up to 30% -50% in the 2nd trimester and 60-90% in the 3rd trimester) [2, 4]. On the other hand, the severity of congenital toxoplasmosis (CT) depends on the age of the fetus at the time of transmission. Fetal infection can lead to intrauterine death, clinical symptomatology at birth (when transmission occurs during the 1st and/or the 2nd trimester), or subclinical evolution at birth with late-developed cerebral or visual sequelae (transmission during the 3rd trimester) [5]. These highlight the importance of early diagnosis, determination of the infection versus conception time to properly manage the risk of transplacental transmission and initiation of timely treatment.

The diagnosis of acute or latent *T. gondii* infection in pregnant women is usually based on the available serological tests (detection of *T. gondii* specific IgG, IgM, IgA, IgE and IgG based-avidity); however, the results of these tests are not always conclusive in terms of accurate estimation of infection time. The correct diagnosis of acute infection is more challenging especially if the first serological investigations are performed in advanced pregnancy. One reason is that acute toxoplasmosis is asymptomatic in more than 90% of cases, and on the other hand precise assessment of maternal infection versus conception is hampered by the lack of systematic examination [4, 6, 7]. The detection of specific IgM associated with low avidity of IgG antibodies is usually an indication of recent seroconversion [1]. The additional systematic tests performed during pregnancy are recommended to evaluate the kinetics of acute phase-specific antibodies, the maturation of specific IgG avidity and the presence of parasite DNA in amniotic fluid or in maternal blood by PCR testing. National programs of systematic screening in pregnant women have been implemented in a limited number of European countries. Thus, in most countries around the world, suspicion of CT is based on the ultrasound detection of fetal lesions and maternal *T. gondii* infection is not always diagnosed during the first trimester of pregnancy [8, 9].

Reports of epidemiological studies show a considerable variation in the prevalence of *T. gondii* infection from less than 10% to over 90% in different parts of the world and even between different population groups within the same country [10-13]. Moreover, there is limited data about the epidemiology of *T. gondii* infection in pregnant women in Romania [13]. Therefore, the objectives of the present study were to detect a primary maternal infection acquired during pregnancy using standard serological tests and PCR and to estimate the sero-prevalence and potential risk factors in acquiring *T. gondii* infection in Romanian pregnant women.

MATERIALS AND METHODS

Study design and patients

The present study was considered a cross-sectional survey but with observational potential, based on the data of a prospective research conducted between January 2009-December 2013 in Cantacuzino National Research Institute, (currently, Cantacuzino National Medico-Military Institute for Research and Development), Bucharest, Romania. Blood samples were collected from 170 pregnant women visiting two medical centers in Bucharest (Cantacuzino National Research Institute and Colentina University Hospital) for the routine antenatal

investigations, follow-up or medication. Relevant epidemiological and medical information of each pregnant woman who gave their consent to the study was acquired from the mandatory notifications submitted by the clinician or by using a structured standard questionnaire.

Serological investigations

Sera obtained after centrifugation of blood samples (n=340) were screened for the presence of anti-*Toxoplasma* IgM and IgG antibodies by standard enzyme linked immunosorbent assay (ELISA) kits, according to manufacturer's instructions. A second blood specimen was taken at least three weeks after the initial sample from all cases with suspected primary *Toxoplasma* infection. The follow-up serum samples were analyzed by ELISA to detect the kinetics of specific IgG and IgM antibodies, the presence of anti-*Toxoplasma* IgA antibodies, and the avidity of anti- *Toxoplasma* IgG. The results of commercially available IgG avidity ELISA kit were expressed as avidity index (AI) in %. AI values greater than 40% were considered as high avidity and infections acquired before the last 4 months; AI values less than 40% were assessed to indicate a low avidity and possible recent *T. gondii* infections (infections acquired within the last 4 months).

Polymerase chain reactions (PCR)

The PCR assays were performed on DNA isolates from blood samples collected in duplicate, into EDTA tubes, from each pregnant woman (particularly from the *Toxoplasma* seropositive women suspected of primary infection). A volume of 220 µl buffy coat sample, prepared from venous blood, was used for DNA extraction with QIAamp DNA MiniKit. The DNA was resuspended in 100 µl AE buffer, supplied within the kit.

T. gondii 35-repeat *B1* gene and *rep529* (non-coding repeated element in 200-300 copies) were selected as the targets for the detection of the presence of parasitic DNA. The nucleotide sequences for the primers used in the *B1* and *rep529* PCR amplification assays are provided in Table 1. Both PCR protocols for the two targets were optimized and evaluated by testing their sensitivity and specificity to DNA detection in *T. gondii* tachyzoites (unpublished data). PCR reactions were carried out in a final volume of 25 µl PCR mixture consisting of 1.5 U Go*Taq* DNA polymerase, 2 mM $MgCl_2$, 200 µM dNTPs, 0.7 pmol of each primer and 10 µl template DNA. A PCR negative-control represented by sterile PCR-grade water, a positive-control sample of DNA extracted from *T. gondii* tachyzoites RH-strain, and an internal control of human *β-globin* [16] were used in PCR amplifications. The PCR products were analyzed on 1.5% agarose gel stained with ethidium bromide.

Statistical analysis

The recorded data were analyzed by Microsoft Excel and SPSS 20.0. The associations between selected explanatory variables and *T. gondii* infection and the differences between the diagnostic tests were evaluated using the chi-square and Fisher's exact tests. The possible predictors of toxoplasmosis were included in a regression logistic model. The strength of the associations between *T. gondii* infections and potential risk factors were assessed by odd ratios (OR) and their respective 95% confidence intervals (CI). The Student *t* test was selected to compare the mean values of the serological results. The statistical significance of the hypothesis testing was defined by two tailed probability value $p<0.05$.

RESULTS

Serodiagnosis and prevalence of toxoplasmosis

One hundred and seventy pregnant women, mean age ± SD (standard deviation) 30.79 ± 3.81 years (range 18-42 years), were enrolled and screened for *Toxoplasma* infections with routine ELISA IgM and IgG tests in this study. The overall seroprevalence of toxoplasmosis in the participants varied according to the type of specific antibodies. Thus, IgG and IgM *T. gondii* seropositivity were 38.24% (65/170) [95%CI: 30.86-45.61] and 18.82% (32/170) [95%CI: 12.89-24.76], respectively. Out of 170 tested women, 20.59% were diagnosed with chronic toxoplasmosis; in a majority of 67.76%, ELISA results indicated the absence of toxoplasmosis; 17.65% were both IgG and IgM reactive (Table 2) and consequently suspected for a *T. gondii* infection acquired during the current

Table 1. Primer sequences used for *T. gondii* DNA detection

Primer name	Sequence (5′- 3′)	Target name	Amplicon Lenght (bp)	References
TOX4 TOX5	CTGCAGGGAGGAAGACGAAAGTTG CTGCAGACACAGTGCATCTGGATT	*rep529**	529	[14]
B22 B23	AACGGGCGAGTAGCACCTGAGGAGA TGGGTCTACGTCGATGGCATGACAAC	*B1***	115	[15]

** GenBank acc. no. AF146527; ** GenBank acc. no AF179871*

pregnancy (with transplacental passage risk) if the serum specimen was obtained in the first five months of gestation. Furthermore, the overall seropositivity for *T. gondii* acute and chronic infections in relation to stage of gestation was higher in the 1st trimester (Table 2).

Serological confirmations performed after three weeks excluded the possibility of an acute *T. gondii* infection in two of the 170 investigated women (1.2%) which were initially detected with *T. gondii* IgG non-reactive and IgM reactive (Table 2). These cases were considered to be false positive results for toxoplasmosis (*Toxoplasma*-specific IgG and IgA antibodies were negative). All thirty women with both IgM and IgG positive were retested for *Toxoplasma* infection with ELISA IgG, IgM, IgA, and IgG avidity on a second follow-up specimen (Table 3). Six of these 30 (20%) cases had a twofold rise in IgM titer, an insignificant increase of IgG titer, low positive *Toxoplasma* IgA, and low IgG avidity, suggesting a recent *T. gondii* infection, possibly acquired in the last two months. Three out of the six pregnant women were in their last two trimesters of pregnancy and considered with a higher potential risk for infection transmission to the fetus. High IgG avidity results were present in the remaining 24 cases, even in those with relatively high titers of the three types of antibodies. In addition, seventeen out of 24 pregnant women did not show any significant change in IgG values, had low IgM titers, and were found in their 1st trimester of pregnancy (Table 3). These observations suggested a tendency to chronic infections possibly acquired in the peri or anteconceptual period. However, all 30 IgM and IgG positive pregnant women were prescribed Rovamycine. The antiparasitic therapy was essential in ten women who were found in their last two trimesters of pregnancy because the risk of toxoplasmosis transmission increases with gestational age.

***B1* and *rep529 T. gondii* PCR amplifications**

PCR analysis was performed for all the 30 selected pregnant women who needed further analysis to confirm with certainty the status of their *T. gondii* infection (six IgM positive women with low IgG avidity and 24 IgM positive women with high IgG avidity) (Table 3). It should be noted that for eight women with positive *T. gondii* IgM and IgG (of which three

Table 2. Combination of ELISA *T. gondii* IgG and IgM antibodies results in pregnant women (%)

Serological status	Total n=170	Stage of pregnancy		
		1st trimester	2nd trimester	3rd trimester
IgG positive and IgM negative	35 (20.59)	27 (15,88)	7 (4.118)	1 (0.588)
IgG positive and IgM positive	30 (17.65)	20 (11.76)	8 (4.706)	2 (1.176)
IgG negative and IgM negative	103 (65.59)	77 (45.29)	21 (12.35)	5 (2.941)
IgG negative and IgM positive	2 (1,176)	2 (1.176)	0	0
Total seropositivity	65 (38.24)	47 (27.65)	15 (8.824)	3 (1.765)
Total seronegativity	105 (67.76)	79 (46.47)	21 (12.35)	5 (2.941)

Table 3. Results of prenatal diagnosis, infection stage and estimated time of maternal infection in the 30 pregnant women suspected of *T. gondii* primary infection

Serological confirmation results			Sero-positive cases (%)	PCR results	*T. gondii* infection stage	Estimated time of maternal infection
IgG, IgM ELISA	IgA ELISA	IgG AIs		DNA positive cases (%)		
IgM ↑↑ + IgG ↑ +	IgA +	Low	6 (20.0)	4(13.3)	Acute	During pregnancy
IgM ↓ + IgG ↑↑ +	IgA ↑ +	High	7 (23.3)	0	Subacute	Possible periconceptual
IgM ↓↓ + IgG ↓ +	IgA -	High	17 (56.7)	0	Chronic	Before conception

with low IgG avidity), Rovamycine therapy was initiated 2 to 14 days prior to collection of blood samples for PCR testing. Both *B1* and *rep529* PCR protocols detected *Toxoplasma* DNA in four of the six (66.67%) cases of interest with IgM, IgA positive antibodies and low IgG avidity. In these pregnant women (two in their 1st trimester, and another two in their 2nd and 3rd trimester, respectively) positive PCR results confirmed the ELISA IgG avidity test and certified a *T. gondii* infection recently acquired during pregnancy with high transplacental transmission risk.

In contrast, negative PCR results in all the 24 women with high IgG avidity confirmed a chronic toxoplasmosis with persistent false-positive *T. gondii* IgA and/or IgM antibodies (Table 3). In this context, the comparisons between the results of IgG avidity and PCR tests revealed a statistically significant relation ($p<0.01$, Fisher's exact test). No imagistic evidence of fetal infections was reported in all high-risk cases, and monitoring during pregnancy was recommended. The exception was a case of primary infection acquired in the first trimester and confirmed by the PCR, where the abortion decision was taken. The estimated time of *T. gondii* maternal infection is shown in Table 3.

Univariate logistic regression analysis used to determine a possible relationship between the seropositive and seronegative pregnant women and possible epidemiological risk factors linked to *T. gondii* infections (Table 4) revealed a statistically significant difference only in situations of undercooked or raw meat consumption ($P<0.05$). Of the six IgM, IgA positive women with low IgG avidity, 83.3% had a history of exposure to *T. gondii* infection by raw meat consumption (this percentage included all PCR positive cases).

DISCUSSION

Early serological determination of *T. gondii* specific antibody status in a pregnant woman (ideally performed between 8-13 weeks of gestation) allows appropriate prophylactic or therapeutic measures in the case of primary infection acquired during pregnancy and prevents a congenital transmission to fetuses.

In Romania, unlike other countries where a nationally subsidized program and guidelines for the primary prevention of congenital toxoplasmosis have been implemented [8], serological screening in pregnancy is not mandatory and testing is performed frequently in suspicious cases. In the present study, the routine serological diagnosis based on the ELISA assay showed a high sensitivity, but the specificity of the test for *T. gondii* IgM antibodies detection varied among tested women. Thus, interpreting of positive IgM alone or in combination with seroconversion of *T. gondii* IgG and even in the presence of a positive IgA should be done with caution. There have been reports of persistent IgM for two years, and persistent IgA over 3-4 months following an acute infection [17].

The subsequent measurement of antigen binding avidity of the *T. gondii* IgG antibodies allows a precise definition of the time of the infection, can confirm the IgM diagnosis by the presence of a low IgG AI, but it can raise debates in the case of high IgG AI results. High IgG avidity rules out an acute infection (mainly in early pregnancy or close

Table 4. Univariate analysis of five potential predictors of toxoplasmosis in 170 pregnant women

Variable	Total	% IgG positive	P-value	OR (95%CI)
Age range (years)				
18-30	33	41.25	Referent	Referent
31-42	32	35.56	0.545	0.78 (0.42-1.46)
Residency				
Urban	58	36.94		
Rural	7	53.85	0.395	1.68 (0.56-5.06)
Education level				
Tertiary	44	35.77		
Primary ± secondary	21	44.68	0.372	1.45 (0.73-2.87)
Raw or undercooked meat consumption				
No	31	25.83		
Yes	34	68.00	<0.0001	6.10 (2.96-12.55)
Contact with cat				
No	54	35.76		
Yes	11	57.89	0.079	2.47 (0.93-6.51)

to conception), but when it is associated with significant increase in *T. gondii* IgG levels may also suggest a reinfection or reactivation [4, 17, 18]. In the present study, the determination based on the kinetics of the specific IgM, IgG and IgA antibodies as well as evaluation of the maturation of the *T. gondii* IgG response did not exclude the potential of recently acquired infections during pregnancy in 20% or possibly periconceptionally acquired infections in 23.33% of women (Table 3). Periconceptional seroconversions rarely lead to congenital infections and are thought to be the result of a delayed parasite migration through placenta to the fetus from weeks to months post infection [4, 19, 20]. In addition, the fact that 80% of women suspected of primary infection in the present study had stable *T. gondii* IgG avidity does not exclude the possibility of a variable maturation of IgG in relation to the kinetics of the immune response. In this sense, there are scientific reports of a persistent low *T. gondii* IgG avidity for 5 to 14 months in pregnant women [21, 22].

The PCR potential for the diagnosis of active toxoplasmosis is of great importance for the detection of congenital toxoplasmosis, particularly when the serological results are not sufficiently clear to establish the status of the infection. Making abstraction of the few pregnant women detected with low *T. gondii* IgG avidity as well as the presence of potential confounding factors that have influenced the PCR results (i.e. antiparasitic treatment started before sample collection), the protocols used in the present study exhibited a general sensitivity of 66.66% for the detection of *T. gondii* DNA isolated from maternal blood samples. The failure to detect *T. gondii* tachyzoite DNA in two of the suspected primary infection cases may have three potential causes. (1) One of them could be associated with the relatively significant difference ($P = 0.034<0.05$, $t = 2.776$) seen between the lower IgG AI values in the 4 positive PCR cases (6-25% index range) and the higher IgG AI values in the two negative cases by PCR (29-33% index range). This observation supports the idea of variable maturity of the specific IgG response and implicitly the existence of a different parasitic charge in the blood. (2) Another more likely explanation is related to the implications of Rovamycine therapy administered 2-14 days before EDTA blood sample collection in 3/6 of low IgG avidity pregnant women. Rovamycine has a destructive effect on circulating tachyzoites,

reduces parasitic multiplication ability, but it has no effect on tissue cysts. Consecutive, antiparasitic treatment leads to a rapid elimination of the parasite from blood, DNA degradation, and false negative PCR results [7, 23]. The gap between treatment initiation and sampling collection varied inversely with the chance of PCR amplifications. Thus, the amplification of the *T. gondii* DNA was successful when the sample was most recently collected, respectively two days after initiation of treatment with Rovamycine. Both PCR protocols for two genomic targets used here for parasite DNA detection were equally affected by antiparasitic therapy. (3) On the other hand, the time of *T. gondii* DNA persistence in immunocompetent pregnant women is extremely important. In patients with acute toxoplasmic lymphadenopathy, the "clearance" time for *T. gondii* DNA is estimated to be between 5.5 and 13 weeks from the first day of infection [6, 24] and the kinetics of circulating tachyzoites may be fluctuating. Thus, PCR detection of parasitic DNA in maternal blood may suggest a more recent infection with an average of two months or an apparent parasitemia. In the light of these considerations, in the absence of antiparasitic prophylaxis prior to sampling, a negative PCR result in pregnant women with low IgG avidity does not exclude the possibility of an acute infection because the parasitemia in peripheral blood is either of short duration and intermittent or low. However, detection of parasitic DNA in maternal blood only serves to confirm an infection recently acquired during pregnancy with transplacental transmission risk but it does not confirm a congenital toxoplasmosis acquired by the fetus. For these reasons, PCR performed on amniotic fluid collected at 18 weeks of gestation is the most appropriate method for the prenatal diagnosis of congenital toxoplasmosis [6, 25, 26].

Determining the gestational age during which maternal infection occurs is also very important. In the present study, most of the pregnant women (75.29%) were tested in their 1st trimester and the infections were staged as chronic in 25.88% and acute in 1.76%.

It is known, that the rate of transplacental infection increases and severity of disease in the fetus decreases as pregnancy approaches the term [27]. This explains why, in case of first-trimester infection, the decision on therapeutic abortion can be taken.

In the present study, the overall *T. gondii* seroprevalence detected in pregnant women was 38.24%. This finding was in the range of sporadic previous seroprevalence reports between 1996-2009 from different areas of Romania (15.8-57.6%) [13], but was lower than the *T. gondii* prevalence recently reported among childbearing women in Western Romania (51.89%) [28]. These observations suggest that toxoplasmosis in pregnant women remains a parasitosis of national concern. This is partly due to the low level of knowledge regarding prevention measures for this parasitosis with the risk of transplacental transmission in pregnant women. A National Romanian survey showed that 68.7% pregnant women seem to have heard about toxoplasmosis, and only 35% know the principal way of getting toxoplasmosis [29]. Epidemiological evidence presented in our study but also reported in other national and international studies [6, 30-34] is the significant association between raw and uncooked meat consumption and *Toxoplasma gondii* infection. In Romania, the potential risk of human infection with *T. gondii* was highlighted by the consumption of raw pork, especially meat derived from backyard pigs [35].

CONCLUSIONS

Serological investigations used in the screening or confirmation of a primary *T. gondii* infection that occurs in pregnant women are not sufficient under conditions of persistent specific positive IgM or IgA antibodies or low IgG avidity. Corroborating PCR and serological data is the safest formula for determining the moment when a primary *T. gondii* infection occurs in pregnant women. The relatively high seroprevalence of *T. gondii* infection in pregnant women in Romania implies the need to implement an educational information program, systematic surveillance and screening programs to reduce the negative impact on the conception product in the case of primary infection as well as additional epidemiological studies to determine the economic impact of this parasitosis.

Conflict of interests: Authors declare no conflict of interests.

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