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Prenatal toxoplasmosis diagnosis from amniotic fluid by PCR

Diagnóstico pré-natal da toxoplasmose no líquido amniótico através da técnica de PCR

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Abstract *Toxoplasmosis is one of the most common infections all over the world. Most cases are asymptomatic, except in immunosuppressed individuals and fetuses, which can be seriously damaged. Prenatal diagnosis should be made as soon as possible since treatment of the mother can minimize fetal sequelae. Our aim in this study was to test the polymerase chain reaction technique (PCR) in 86 samples of amniotic fluid from women who seroconverted during pregnancy. DNA was amplified using external primers and, in a second step, internal primers, in a nested PCR system. Samples were also inoculated into mice and the newborn were evaluated by* T. gondii *serology, skull x-ray, transfontanel ultrasound, fundoscopic examination, lumbar puncture and clinical examination. PCR was positive in seven cases and negative in 79. Among PCR-positive cases, two were negative by inoculation into mice and by clinical evaluation; among PCR-negative ones, three had clinical evidence of toxoplasmosis and one was positive after inoculation into mice. PCR showed values of sensitivity = 62.5% and specificity = 97.4%; the values of inoculation into mice where 42.9% and 100%, respectively. Although PCR should not be used alone for prenatal diagnosis of congenital toxoplasmosis, it is a promising method and deserves more studies to improve its efficacy.*

Key-words*: Congenital toxoplasmosis. PCR. Amniotic fluid.*

Resumo *A toxoplasmose é infecção freqüente em todo o mundo, mas na maioria dos casos não traz repercussões importantes para o paciente, exceto indivíduos imunodeprimidos e fetos, os quais podem apresentar graves seqüelas. O diagnóstico precoce durante a gravidez é altamente desejável, já que o tratamento da gestante reduz a freqüência e gravidade da infecção fetal. Neste estudo aplicou-se a técnica de PCR em 86 amostras de líquido amniótico de gestantes que apresentaram soroconversão durante a gravidez. O DNA foi amplificado usando-se iniciadores externos e internos, num sistema de nested PCR. As amostras foram também inoculadas em camundongos e os recém-nascidos acompanhados clinicamente através de sorologia, RX de crânio, ultrassom transfontanela, exame de fundo de olho e punção lombar. Pela PCR, sete casos foram positivos e 79 negativos. Entre os positivos, dois não se confirmaram pela inoculação em camundongo nem pela avaliação clínica da criança; dos negativos, três apresentaram clínica de toxoplasmose congênita, e em um deles o exame de inoculação em camundongo foi positivo. A sensibilidade e especificidade da PCR foram 62,5% e 97,4%, respectivamente; a inoculação em camundongos mostrou 42,9% de sensibilidade e 100% de especificidade. Embora a PCR não deva ser usada como único teste diagnóstico da toxoplasmose congênita, trata-se de método promissor e merece mais estudos para aumentar sua eficácia.*

Palavras-chaves*: Toxoplasmose congênita. PCR. Líquido amniótico.*

Congenital toxoplasmosis is the result of transplacental transmission of *Toxoplasma gondii* from an acutely infected mother. The risk of fetal infection varies according to the gestational age at the time of maternal infection. When this occurs in the 11-14 weeks of gestation, the incidence of fetal infection is 7.2%, whereas in the 31-34 weeks period it is 67%; the overall rate of fetal infection is 7.4%[12].

Congenitally infected infants can present, at birth, the classical signs and symptoms of congenital toxoplasmosis, such as hydrocephalus, chorioretinitis, cerebral calcifications and stroke, or they can be symptom-free and develop long-term sequelae during childhood or adult life[15].

Toxoplasmosis can be diagnosed by: 1) serological profile, although this may be unreliable in those who

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are immunodeficient, as in the fetus; 2) isolation of the parasite by animal inoculation or cell culture, which is time consuming; 3) demonstration of the parasite genome by polymerase chain reaction (PCR) technique, a promising method due to its reported good sensitivity and high specificity[12].

Early anti-parasitic treatment of infected mothers has been shown to both decrease the transmission rate to the fetus and to reduce sequelae when intrauterine infection has already occurred[17]. Since the choice of anti-parasitic treatment depends on whether or not the fetus is infected, early detection of the parasite is very important[10].

The aim of this study was to apply the PCR technique to amniotic fluid for the prenatal diagnosis of congenital toxoplasmosis and to compare these results to the postnatal clinical diagnosis.

PATIENTS AND METHODS

We studied 86 amniotic fluid samples from women in whom acquired *T. gondii* infection during pregnancy has occurred. The infection was detected during a routine serologic screening program performed in the Prenatal Unit of Hospital das Clínicas da Universidade Federal de Minas Gerais (HC-UFMG) and Santa Casa de Misericórdia de Belo Horizonte, Minas Gerais. Initially serology (ELISA, ELIFA, immunofluorescence) was performed at each gestation trimester. In the case of a positive result, it was then performed montly. For diagnosis of fetal infection, part of the amniotic fluid was submitted to PCR and the other part was inoculated into mice. After birth, the infants were followed periodically by the infectologist staff of HC-UFMG, and *T. gondii* serology, skull x-ray, transfontanel ultrasound, fundoscopic examination, lumbar puncture and clinical evaluation were performed. Congenital infection was confirmed (clinical signs and/or serology) or discarded (clinical signs absent and negative serology) after at least six months of follow-up.

DNA extraction. From the samples of amniotic fluid, 1mL was used for DNA extraction. This consisted of centrifugation at 5,000rpm for 10 minutes followed by discharge of 800μL of the supernatant. The 200μL left were incubated at 100°C for 15 minutes; 450μL of ethanol and 20μL of ammonium acetate were then added and the tube was kept at -80°C for 60 minutes or at -20°C overnight. Then the sample was centrifugated at 15,000rpm at 4°C during 30 minutes, the supernatant was discharged and the pellet was ressuspended in 50μL of deionized water.

***T. gondii* DNA amplification.** PCR was performed using two pairs of primers that anneal to gene B1[2] of the *Toxoplasma gondii,* leading to amplification of 690 and 178 bp fragments (Table 1). The reaction mixture for the first amplification consisted of: 1) 10μL of the former DNA suspension; 2) 25pmol of each outer primers 1 and 4; 3) 3μL of dNTP 2mM (dATP, dTTP, dGTP, dCTP); 4) 1.5U of Taq polymerase; 5) 2μL of 10X PCR buffer (15mM $MgCl_2$, 500mM KCl, 100mM Tris-HCl pH=8); 6) deionized water to get a final volume of 20μL. DNA amplification was performed in a MJ Research MiniCycler™ (25 cycles of one-minute-denaturation at 95°C, one-minute-annealing at 55°C, 1.5- minute-extension at 72°C and at the end of the program a seven-minute-final-extension at 72°C).

Table 1 - Primers used in polymerase chain reaction.

Primer	Sequence	Product size	Target region
Outer 1 (upstream)	5´ TGGGAGAAAAAGAGGAAGAGA 3´	690bp	position 565
Outer 4 (dowstream)	5´ TTCACTCCATCTCTCGTCTTC 3´		position 1254
Inner 2* (upstream)	5´ AAAGGAACTGCATCCGTTCAT 3´	178bp	position 691
Inner 3* (dowstream)	5´ GACCACGAACGCTTTAAAGAA 3´		position 868

* According to Burg *et al* [1]

After the first amplification, 1μL (10x diluted) of the product was reamplified, now using inner primers 2 and 3. The amplification program was the same, except for the annealing temperature (60°C).

All amplification reactions contained a positive (one microliter of DNA extracted from a *T. gondii* culture) and negative (10μL DDW) controls. Products were submitted to electrophoresis and analyzed in silver-stained 6% polyacrilamide gel.

Mouse inoculation. 2-5mL of the amniotic fluid were centrifugated at 2,000rpm during 10 minutes; after discharging the supernatant, 1mL of PBS (phosphate buffer solution) was added to the pellet and the product was inoculated intraperitoneally into two mice. The animals were clinically evaluated daily; when death occurred, the peritoneal liquid was analyzed at the microscope (looking for tachyzoites). If after 30 days the animals were still alive, they were sacrificed; serology for *T. gondii* was performed and the brain was examined for tissue cysts.

Statistical analysis were performed in Epi-Info Software[5] Version 6.04, using Chi-square (comparison of frequencies), ANOVA (comparison of means) and Kruskal-Wallis (comparison of medians) tests, depending on the data profile and distribution.

RESULTS

From the 86 cases of acute gestational toxoplasmosis studied, maternal-fetal transmission occurred in 8 (9.3%). Clinical manifestations of the infected newborns were variable, as shown in Table 2.

Table 2 - Clinical data in newborns with congenital toxoplasmosis.

	GA		Apgar		Skull		Cerebrospinal	Fundoscopic
Case	(weeks)	Sex	score 1'	Score 5'	X-ray	TFUS	fluid	examination
1	37	M	7	8	calc	hydrocephalus	protein	chorioretinitis
2	37	F	-	7	calc	hydrocephalus	protein	chorioretinitis
3	39	M	9	9	normal	hydrocephalus	NR	normal
4	39	F	9	10	calc	normal	normal	normal
5	25	F	-	-	nP	NP	NP	NP
6	35	F	1	7	calc	NP	normal	normal
7	39	M	1	1	calc	hydrocephalus	protein	chorioretinitis
8	38	F	9	9	calc	normal	normal	chorioretinitis

GA = gestational age; protein = high protein level; calc = intracranial calcification;

TFUS = transfontanel ultrasound; NP = not performed; chorioretinitis = chorioretinitis scar

Mean mother's age in the group with congenital infection was 19.2 ± 3.0 (median = 19 years) and in the group without congenital toxoplasmosis was 23.9 ± 5.4 (median = 24 years) (p = 0.02). There were no difference in the mother's gestational history, gestational age (median = 37.5 weeks in the group with congenital infection and median = 38 weeks in the group without infection, p = 0.21) and newborn gender (p = 0.26). Mean birth weight was lower in the infected group (mean = 2,236.2 ± 922g; median = 2,470g) than in non-infected one (mean = 3,023 ± 495g; median = 3,045g) (p = 0.01). One minute-Apgar score showed similar medians in the two groups (8 and 8; p = 0.49); however, five minute-Apgar score was statistically different (medians 8 and 9 in the infected and non-infected groups, respectively; p = 0.04) (Table 3). Except for Apgar scores, the missing data (which could not be obtained from the records available) were less than 10%, meaning good statistical confidence.

Prenatal exams. In five of eight cases of congenital infection, prenatal ultrasound (US) showed some alteration (four hydrocephalus, two ventricular dilatation, two hepatic calcifications and one intracerebral calcifications and restricted intrauterine growth). In four cases from the group without congenital toxoplasmosis, some changes were detected by US (hepatic and intracerebral calcifications, hydrocephalus and polyhydramnios). US has shown values of sensitivity = 62.5% ($CI_{95\%}$ 25.9-89.8), specificity = 94.8% ($CI_{95\%}$ 86.5-98.3), positive predictive value = 55.6% ($CI_{95\%}$ 22.7-84.7), negative predictive value = 96.1% ($CI_{95\%}$ 88.1%-99.0) (Table 7).

Table 3 - Mother's and newborn's profile in groups with and without congenital toxoplasmosis.

	Mean/median (frequency)					
Variable	toxo + (n=8)		toxo – (n=78)		Missing data (%)*	p-value
	n	(%)	n	(%)		
Mother						
age (median; years)	19		24		2.3	0.02
marital status						
single	3	3.8	22	28.2		
married	3	3.8	36	46.1	9.3	0.74
other	2	2.5	12	15.3		
pregnancies (median)			1	1	2.3	0.80
deliveries (median)			0	0	2.3	0.65
miscarriages (median)			0	0	2.3	0.30
Newborn						
gender						
male	2	2.3	41	48.8		
female	5	5.9	36	42.8	2.3	0.26
birth weight (median)			2,470g	3,045g	0.0	0.01
gestational age (median)			37.5 weeks	38 weeks	5.8	0.21
Apgar 1' (median)			8	8	20.9	0.49
Apgar 5' (median)			8	9	20.9	0.04

* Percentage of cases in which the data could not be obtained from the records available

Amniotic fluid PCR was positive in five of the eight cases of maternal-fetal transmission (three false-negative cases). In the group without fetal transmission, PCR was positive in two (two false-positives) (Table 4). Sensitivity = 62.5% ($CI_{95\%}$ 25.9-89.8), specificity

Table 4 - PCR with amniotic fluid for diagnosis of congenital toxoplasmosis.

	Amniotic fluid PCR		
PCR	toxoplasmosis +	toxoplasmosis -	total
Positive	5	2	7
Negative	3	76	79
Total	8	78	86

= 97.44% ($CI_{95\%}$ 90.2-99.6), positive predictive value = 71.4% ($CI_{95\%}$ 30.3-94.9), negative predictive value = 96.2% ($CI_{95\%}$ 88.6-99) (Table 7). In the positive PCR samples (five correct results and two false-positive ones), only in one case the result was achieved after the first amplification; in all the others the second step was necessary. This occurred when the same pair of primers (inner) was used for both amplifications or when the outer and inner (nested PCR) system was applied.

Amniotic fluid inoculation in mice was performed in seven cases in the group with congenital toxoplasmosis; the results were positive in three and negative in the other four cases. There was no false-positive result (Table 5). Sensitivity = 42.9% ($CI_{95\%}$ 11.8-79.8), specificity = 100% ($CI_{95\%}$ 92.4-100), positive predictive value = 100% ($CI_{95\%}$ 31.0-100), negative predictive value = 93.7% ($CI_{95\%}$ 83.7-97.9) (Table 7).

When PCR was associated to mouse inoculation the results were slightly different (Table 6): sensitivity = 75% ($CI_{95\%}$ 35.6-95.5), specificity = 96.6% ($CI_{95\%}$ 87.3-99.4), positive predictive value = 75% ($CI_{95\%}$ 35.6-95.6), negative predictive value = 96.6% ($CI_{95\%}$ 87.3-99.4) (Table 7).

Table 8 shows a comparation between prenatal and postnatal exams and the final clinical diagnosis of congenital toxoplasmosis.

Table 5 - Amniotic fluid inoculation into mouse for diagnosis of congenital toxoplasmosis.

	Mouse inoculation		
Inoculation	toxoplasmosis +	toxoplasmosis -	total
Positive	3	0	3
Negative	4	59	63
Total	7	59	66

Table 6 - Combination of amniotic fluid PCR and mouse inoculation for diagnosis of congenital toxoplasmosis.

	Association of PCR and mouse inoculation		
PCR+inoculation	toxoplasmosis +	toxoplasmosis -	total
Positive	6	2	8
Negative	2	57	59
Total	8	59	67

Table 7 - Comparison between prenatal tests for diagnosis of congenital toxoplasmosis.

Test	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Ultrasound	62.5	94.8	55.6	96.1
PCR	62.5	97.4	71.4	96.2
Inoculation	42.9	100.0	100.0	93.7
PCR + inoculation	75.0	96.6	75.0	96.6

PPV = positive predictive value; NPV = negative predictive value; inoculation = inoculation in mouse

Table 8 - Comparison between pre and postnatal exams and the final diagnosis of congenital toxoplasmosis.

Case	Ultrasound	PCR	Mouse inoculation	Newborn IgM	Toxoplasmosis
1	+	+	+	+	+
2	+	+	+	-	+
3	-	+	-	-	+
4	-	+	-	-	+
5	+	+	NP	NP	+
6	+	-	-	-	+
7	+	-	+	NP	+
8	-	-	-	-	+
9	-	+	-	-	-
10	-	+	-	-	-
11-86*	*	-	-	-	-

*Patients 11 to 86 were PCR, inoculation, serology and clinically negative. Patients 11, 42, 43 e 74 had some ultrasound alteration.

NP = not performed

DISCUSSION

Congenital toxoplasmosis, an worldwide infection, can vary from asymptomatic to serious sequels. Prevention of neonate damage can be attained by information of the mothers, treatment of pregnant women already infected and early treatment of the newborns. Treatment is known to reduce the transmission rates and clinical manifestations[9]. The earlier the diagnosis is made, the better the outcome.

The maternal-fetal transmission rate found in this study was 9.3%, higher than 7.4% obtained by Hohlfeld et al[12] and lower than 38.7% and 29% from Foulon *et* al[8] and Dunn et al[6], respectively. However, we could not determine the transmission rate according to the period of maternal seroconversion, which can vary widely.

There are many studies describing the use of PCR to diagnose toxoplasmosis, using P30 gene[2], ribosomal DNA[3] or TGR1E[4] (a repetitive original DNA sequence) as target for amplification. In our study, using B1 gene as target[1], we obtained 62.5% of sensitivity and 97.4% of specificity (two false-positive and three false-negative results), values lower than those achieved by Hohlfeld et al[12], Cazenave et al[3] and Gratzl et al[11]. Similar results were the sensitivity of 76.2% of Gangneux et al[16] and 55% of Jenum et al[13].

The two false-positive cases could be explained: 1) PCR can detect *T. gondii* DNA already damaged by drugs used in the treatment, which is unable to infect mouse, cell culture or the fetus; 2) although all measures were taken to prevent sample contamination, this can not be ruled out; 3) after birth, children were clinically followed for at least six months, when treatment was interrupted and patients were not seen anymore. Since there are cases of sequels appearing 15-20 years later, we can be misdiagnosing some cases[14]; 4) a slight possibility is the contamination of the sample by maternal blood.

To improve sensitivity the number of amplification cycles can be increased, but this raises the risk of contamination. In the same way, to improve specificity amplification can be done in two steps, although this also increases contamination and, therefore, false-positive results.

The three false-negatives results could be explained by: 1) short time between maternal infection and amniocentesis, fetal transmission occurring after amniocentesis; 2) inadequate storage of the samples and DNA degradation; 3) although B1 gene is repeated 35 times in the toxoplasma genome, PCR was usually performed in 1mL of amniotic fluid, which might not be representative; 4) the presence of cells, human DNA and especially blood can interfere in PCR reaction[17]. Aiming to rule out PCR inhibition, we submitted all samples to a phenol-chloroform extraction; the results, however, were not different (data not shown).

Amniotic fluid mouse inoculation gave 42.9% of sensitivity and 100% of specificity, results lower than those obtained by Foulon et al[8] (58% and 98%) and Gangneux et al[16] (52.9% and 100%). Mouse inoculation is a good test, but since the presence of viable parasites is necessary for mice infection, delay in inoculation, problems in storage or even treatment of the mother before amniocentesis can interfere in the results.

PCR and mouse inoculation are different methods that have different performances. In this study, the combination of both tests slightly improved sensitivity (75%) from 62.5% for PCR alone and 42.9% for mouse inoculation alone.

Taken together, our results showed that a negative prenatal diagnosis based on PCR alone does not always exclude the possibility of congenital infection, as well positive results do not always confirm it. Clinical evaluation of the newborns during the first year of life is obviously necessary. The final diagnosis of congenital toxoplasmosis should be based on clinical data, serological evaluation, PCR and mouse inoculation.

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