

Optimizing the parasitological diagnosis of congenital toxoplasmosis

Otimizando o diagnóstico parasitológico da toxoplasmose congênita

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Abstract

Early diagnosis of Congenital Toxoplasmosis is highly important, since it can make the treatment possible and reduces sequela for the infant. Serological diagnosis alone cannot be accurate when it does not identify the IgA, IgM or IgG antibodies of low avidity, which do not cross the placental barrier. Therefore, parasitemic identification is important to be carried out by demonstrating the parasite in the peritoneal exsudatum of mice inoculated with suspected biological material, however such method is little sensitive and too much time-consuming. This research study aims at optimizing mice inoculation through serological screening, and encephalic histopathology, in order to identify whether there was contamination. Out of 138 fetuses and/or newborn samples taken from pregnant women positive for active toxoplasmosis inoculated intraperitoneally in mice, only 5 showed positive through parasite demonstration in the peritoneal exsudatum. Histopathology showed the agent in 45 cases and in 67 the presence of anti-toxoplasma antibodies in the mice bloodstream by using indirect immunofluorescence technique. Mice serology and encephalic histopathology in addition to reducing the amount of time necessary for the outcomes from 120 to 60 days increased the positivity of 3.6% to 50.4% and 33.8%, respectively.

Key words: Congenital Toxoplasmosis, Parasitological Diagnosis, Mice Inoculation, Experimental Serology.

Resumo

O diagnóstico precoce da toxoplasmose congênita é de fundamental importância, pois possibilita o tratamento e redução das sequelas que esse pode provocar ao conceito. O teste sorológico isolado não permite o diagnóstico quando não identifica os anticorpos das classes IgA, IgM ou IgG de baixa avididade, que não atravessam a barreira placentária. Por isso, a identificação da parasitemia é importante sendo feita pela demonstração do parasito no lavado peritoneal de camundongos inoculados com materiais biológicos suspeitos, mas esse método é pouco sensível e demorado. Assim esse estudo tem como objetivo otimizar o diagnóstico da toxoplasmose por inoculação em camundongo, através da realização da sorologia do sangue periférico desses, e histopatologia encefálica para identificar a sua contaminação. Em 138 amostras de sangue de fetos e/ou recém nascidos provenientes de grávidas com toxoplasmose ativa inoculadas intraperitonealmente nos camundongos, apenas cinco mostraram-se positivas pela demonstração do parasito no lavado peritoneal. A histopatologia demonstrou o agente em 45 casos e a presen-

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ça de anticorpos antitoxoplasmas na corrente sanguínea dos camundongos pela técnica de Imunofluorescência Indireta foi encontrada em 67 casos. A sorologia e a histopatologia nos camundongos, além de reduzir o tempo da liberação dos resultados de 120 para 60 dias possibilitaram um aumento dos casos positivos de 3,6% para 50,4% e 33,8% respectivamente.

Palavras-chave: Toxoplasmose congênita. Diagnóstico parasitológico. Inoculação em camundongos. Sorologia experimental.

1 Introduction

In Brazil, from 25 to 40% of pregnant women are seronegative for Toxoplasmosis (VAZ et al., 1990; SPALDING et al., 2013). The risk of acute infection during the pregnancy is about 1% and the fetal transmission occurs in 30% of cases, which leads the fetal infection to variable severity (WIRDEN et al., 1999; FRIEDMAN et al., 1999). Congenital infections are among the highest causes of morbidity and mortality in the ante and postnatal period (WALLON et al., 2013). In Goiânia, congenital toxoplasmosis has relevant importance, accounting for 8.9% of incidence (AVELINO et al., 2003).

Due to the fact that 90% of infected fetuses and/or newborns are asymptomatic or oligosymptomatic (WONG; REMINGTON, 1994; WALLON et al., 2013), there are often clinical and laboratorial under diagnoses concerning this disease – which could last many years as so. Congenital transmission occurs when the woman acquires the primary infection during pregnancy and/or the chronical infection reactivation occurs (SANTANA; ANDRADE; MORON, 2003), being variable in severity, depending on the considered location, ranging from 0.2 to 34/1000 deliveries (WILLIAMS et al., 1981; COUTINHO; GARCIA; AMENDOEIRA, 1983; PEDREIRA, 1995; AVELINO, 2000).

The parasite reaches the fetus via placenta causing damage of different severity degrees, depending on the virulence of the parasite strain, the pregnant women's immune response, and which period of pregnancy she is in, which may also result on fetal death or in serious clinical manifestations (DESMONTS et al., 1974; FIGUEIRO FILHO et al., 2012). Those mostly frequent are retinocoroiditis and neurological alterations. In the newborn se-

rum, the high presence of IgG strain antibodies increase or remain positive from one to eighteen months, which could indicate congenital toxoplasmosis, and those that decrease and tend to become negative represent maternal antibodies passively transferred (CAMARGO, 2010). Fetal infection could be attenuated or even prevented when there is maternal treatment right after early diagnosis (WONG; REMINGTON, 1994).

Specific IgM, IgA, IgE, and IgG strains antibodies are produced in response to *T. gondii* infection, being IgM, IgA, and IgE suggestive of recent and/or active infection; and IgG shows chronical infection remaining all life long, concerning the acquired infection (GROVER et al., 1990; CAZENAVE; CHEYROU; BEGUERET, 1993).

The serological techniques commonly used in order to diagnose toxoplasmosis are: Indirect Immunofluorescence (IIF), Immune Absorption (ISAGA), and Enzyme-Linked Immunosorbent Assay (ELISA). These techniques, when applied together, increase the outcome sensitivity, reaching 89.5% of pregnant women (DENMARK; CHESSUM, 1978; SOUNIS, 1979; PRATLONG et al., 1994; CAMARGO, 2010).

Classifying the risky pregnant women in the acute phases of toxoplasmosis is usually carried out by using indirect immunofluorescence trials, being the most available in public laboratories, showing highly practical and accurate; however there is a significant percentage of inconclusive trials (CAMARGO et al., 2010). Nowadays, the tests with filtering paper have been being used to antenatal screening, which has high sensitivity and specificity rate (FIGUEIRO FILHO et al., 2012).

In fetuses and newborns such techniques are inaccurate due to their immune immaturity, and the interference of high levels of maternal antibodies in the fetal bloodstream inhibit IgM formation, thus making it necessary the diagnosis confirmation through parasitological techniques able to show the presence of the parasite in the examined material, which, eventually, indicates active infection, which confirms, according to the trials, congenital transmission (MONTROYA; LIESENFELD, 2004).

Parasitological methods in the chronical phases of toxoplasmosis are of low sensitivity due to the wide spread of its agent and its tropism through different tissues (WONG; REMINGTON, 1993; AMENDOEIRA; COSTA; SPALDING, 1999; REY, 2008).

In the same manner, it is known that parasitemic infection is detectable intermittently in some patients (HOFFLIN; REMINGTON, 1985; FILICE et al., 1993; CRISTO, 2004). Due to the fact that it is an obligatory intracellular parasite, culture *in vitro* is highly costly and it is too much time-consuming for the outcomes, most of the time, being effective in less than 50% of cases. Parasite isolation can be done by using inoculation in mice, which is more sensitive, the long period of observation and maintenance of animals in bioteria presents one of the greatest difficulties in the application of such method (GROVER et al., 1990; HITT; FILICE, 1992; JAMES et al., 1996).

The research protocol to which this study is attached makes use of spiramycin 3g/day dose, applied to all pregnant women with presumed acute infection, which makes it difficult the parasitological confirmation by reduction of parasite titres circulating. It is highly important that doubtful serology to be confirmed through parasitological techniques, being the most commonly used the inoculation of biological material in mice.

This study aimed at optimizing the inoculation technique of human biological material in mice peritoneum for detecting toxoplasma infection. In order to do so, we have assessed encephalic histopathology and serology in mice inoculated with amniotic fluid, fetal blood and peripheral blood, and cephalic rachidian fluid of newborns, taken from pregnant women with active toxoplasmosis.

2 Materials and methods

From September, 2002 to January, 2010, 77 pregnant women were followed up in the Hospital and Clinics (Federal University Hospital) and referred to antenatal treatment for showing serological screening positive for toxoplasmosis compatible with acute infection (IgM positive or IgG of low avidity in previously seronegative patients). Serological screening was identified by using MEIA technique (Microparticle Enzyme Immunoassay).

Amniocenteses and cordocenteses were carried out in the patients taking 50 samples of amniotic fluid and 65 from fetal blood. 11 samples of peripheral blood were taken from the newborns, and 12 of liquor, scoring 138 samples as a whole.

The amniotic fluid, liquor, and blood (fetuses and newborns) were centrifugated at 3,000 RPM, for 10 mi-

nutes, the red blood cells sediment inoculated via peritoneum with 1 ml syringe in a set of 3 mice (0.2 mL/animal). During the animals follow up, the arising of clinical signs such as hair raising, weight loss, and lethargy, were considered as a sign of infection, being later on slaughtered by cervix disruption, then peritoneal exsudatum was taken out in order to search for tachyzoite antigens forms.

In the mice showing no apparent signs of infection after 60 days of the first inoculation, the blood was collected by their heart puncture, then they were slaughtered and necropsy was carried out. From those infected and non-infected animal blood samples, after serum collection, serology was carried out so that IgG and IgM antibodies detection could be made through IIF. Encephalon was split into two parts, one of them was smashed, and used for a second inoculation, and the other one was fixed in alcohol and later on put into formol, with which histological lamina were prepared. The same procedure described previously was carried out with the mice inoculated later, being slaughtered 60 days after that.

Indirect immunofluorescence – IIF was used (CARMARGO 1974), for detecting *anti-T. gondii* antibodies, the antigen used was RH strain, kept in the IPTSP-UFG laboratory (laboratório de Biologia, Fisiologia e Imunologia dos Protozoários de Interesse Humano). IIF was carried out with sera diluted in series, from 1:5 for IgM detection, and 1:10 for IgG. Anti-IgM and anti-IgG conjugated were used in 1:200 dilution, (isothiocyanate of fluorescein labelled SIGMA).

Histopathological lamina were prepared with encephalon fragments 5 mm thick and fixed in alcohol 70% for 24 hours, and transferred to tamponated formol at 10%, dehydrated in alcohol in increasing concentration amount, diafanized in xylol, and embedded and blocked with hot paraffin. 10 histological cuts of 5 µm thick were carried out in each experiment. Dye used was hematoxylin eosin-HE (JUNQUEIRA L. C.; JUNQUEIRA L. M., 1983).

This study was approved by the Animal and Human Research Ethics Committee (Comitê de Ética em Pesquisa Humana e Animal) and protocolled in CE-OMHA/HC/UCG under the registry number of 039/02, as well as the Term of Agreement (Termo de Consentimento Livre e Esclarecido) of the material donors used in this study, in accordance with the current ethical principles.

3 Results

Out of 138 samples, 115 were taken from pregnant women (65 of fetal blood, and 50 of amniotic fluid), and 23 from newborns (11 of peripheral blood, and 12 of cephalic rachidian fluid).

The parasite was isolated through peritoneal exsudatum in only 3.6% (5/138) samples, 2 of them were of fetal blood (FB), 2 of amniotic fluid (AF), and one of peripheral blood of newborns (NB). Sera and encephala obtained from inoculated mice with the other 133 samples were assessed through IIF and HE, respectively, for the infection to be confirmed.

Through IIF, in the first inoculation mice blood samples it was possible to detect 50.4% (67/133) of serological positivity, and in second inoculation blood samples 47.4% (63/133).

Through histopathology of mice encephala in the first inoculation, the parasite was found in 22.6% (30/133) samples, in the lamina of the second inoculation, 13.5%

(18/133) were positive, three of them detected in both inoculations, with a total of 33.8% positive cases.

In all mice positive through histopatolgy, the infection was confirmed through IIF, and in 13.0% (18/138) the infection was detected only through IIF, thus, showing higher positivity.

The animals inoculated with liquor showed higher percentage of seroconversion with antitoxoplasma antibody detection in 66.6% (8/12) samples. The amniotic fluid showed parasite identification in 56.0% (28/50), fetal blood showed 43.1% (28/65) of positivity, and newborn blood showed 27.3% (3/11).

36.9% (24/65) of mice inoculated with fetal blood showed positive for the parasite in encephala histopathological cuts, 33.3% (4/12) of liquor, 30.0% (15/50) of amniotic fluid, and 18.2% (2/11) of blood taken from newborns (Table 1).

The outcomes were assessed through Kruskal Wallis non-parametric test.

Table 1 – Congenital toxoplasmosis diagnosis through rats inoculation with fetal blood, amniotic fluid, newborn blood and liquor, compared with experimental encephalic histopathological diagnosis, and experiment IIF.

		FB n = 65		AF n = 50		NBB n = 11		CRF n = 12		Total Score n = 138
		1st Inoc.	2nd Inoc.	1st Inoc.	2nd Inoc.	1st Inoc.	2nd Inoc.	1st Inoc.	2nd Inoc.	Both Inoc.
ISOLATED	n	2	0	2	0	1	0	0	0	5
	%	4.6	0	4.0	0	0	0	0	0	3.6
HISTO	n	13	13	12	4	2	0	3	1	45
	%	20.0	20.0	24.0	8.0	18.2	0	25.0	8.3	33.8
IIF	n	28	26	28	27	3	3	8	7	67
	%	43.1	40.0	56.0	54.0	27.3	27.3	66.7	58.3	50.4

Fonte: **FB** – Fetal blood, **AF** – Amniotic fluid, **NBB** – Newborn blood, **CRF** – Cephalic rachidian fluid, **ISOLATED** – Parasite in the peritoneal exsudatum, **HISTO** – Encephalic histopathology, **IIF** – Indirect Immunofluorescence. $P \geq 0,05$ – KW

4 Discussion

Despite serological screening effectiveness in 89.5% pregnant women (DENMARK; CHESSUM, 1978; SOUNIS, 1979; PRATLONG et al., 1994; CAMARGO, 2010), there is no specificity, in regard to fetal and/or infant infection confirmation due to immune immaturity influence (AVELINO et al., 2003). Parasitological methods are essential for the solution of such issues, and inoculation is justified for suspected materials via mice

peritoneum, with further observation of *Toxoplasma gondii* in the peritoneal exsudatum (CAMARGO et al., 2010; KAWAZOE, 1995; FRENKEL, 1997; BEAZLEY; EGERMAM, 1998; MONTOYA; LIESENFELD, 2004).

With immediate onset of treatment for presumed maternal infections, there is a reduction in the parasite rate, and such methodology has been showing low sensitivity (HOHLFELD et al., 1989; FOULON; NAESSENS; DERDE, 1994) as reported in this paper, which showed

3.6% (5/138) positivity through direct testing with tachyzoite forms present in peritoneal exsudatum of inoculated mice. Notwithstanding, the analysis of mice inoculated with human material through encephalic histopathology showed the parasite in 33.8% (45/133) of cases, showing higher positivity.

Seroconversion was reported in 50.4% (67/133) samples analyzed through IIF of mice blood, which confirms the parasite presence in the inoculated material, supporting Calvão's data (2002), which indicate IIF as the most recommended method to carry experimental serological screening out. Moreover, the assessed material was likely to show low presence of parasite, due to previous treatment undertaken by pregnant women and/or low virulence rate found in the strains due to parasite intrinsic features which influenced the experimental infection conduct showing the evidence of subclinic disease, making the detection of experimental parasitemia difficult.

The biological material most recommended for detecting *Toxoplasma gondii* infection in infants was liquor which induced to mice seroconversion in 66.6% (8/12) of cases, when assessed through IIF, contrary to Holliman et al. (1994) who indicates peripheral blood as the most recommended material to be assessed. On the other hand, in fetuses the amniotic fluid was the most recommended, with 56.0% (28/50) of positive cases, in accordance with Holthfeld et al. (1994).

The comparative analysis between the first and the second inoculation positive cases, assessed through IIF, showed that in 94.0% (63/67) the outcomes were the same, thus showing that the second inoculation was unnecessary, since it does not add any data in diagnosis terms. Whereas the same analysis between positive cases in both inoculations, assessed through histopathology (HE), showed accordance in only 7.7% (3/39) cases, indicating the importance of the second inoculation, since it increases substantially the test sensitivity.

5 Conclusions

IIF in mice blood showed more efficiency than experimental histopathology which in turn showed the outcomes to be more accurate than those shown through mice inoculation, thus showing itself as a parallel method to experimental inoculation.

The biological material most recommended to infant *Toxoplasma gondii* infection screening was cephalic rachidian fluid, and in fetuses the amniotic fluid.

The second inoculation outcomes assessed through IIF did not show any significant data regarding the diagnosis, thus they can be unnecessary to be done, reducing significantly the animal rate per assay. Whereas that histopathology outcome in the second inoculation showed higher sensitivity to screening its application is highly recommended. Thus IFIs and histopathology techniques differ in the need of the second inoculum in mice when performed alone, however are complementary when applied in parallel.

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