

The investigation of congenital toxoplasmosis in a tertiary care hospital in Turkey

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ABSTRACT

الأهداف: تحديد سبب حدوث داء المقوسات الخلقي في تركيا.

الطريقة: أجريت هذه الدراسة بقسم الطفيليات، بكلية الطب بجامعة إيج – أزمير – تركيا، خلال الفترة ما بين 2006م وحتى 2007م. تم الحصول على الدم عبر الوريد قبل الولادة ودم الحبل السري خلال الولادة من 138 امرأة. كما تم البحث عن وجود الأجسام المضادة (IgG) و (IgM) بواسطة طريقة اليسا، وبواسطة اللطخة الغربية واختبارات مصلية أخرى.

النتائج: لدى اتحاد طريقتي اليسا واللطخة الغربية حساسية كبيرة بين التقنيات المصلية المستعملة، ماعدا عينة واحدة. كانت النتائج للحبل السري ومنطقة الوريد متوافقة مع عدم وجود فرق ملحوظ.

خاتمة: أظهرت البيانات التي تم الحصول عليها أن التصنيف المبكر للأجسام المضادة (IgG) والمصطبغة بواسطة المواليد المصابين خلقياً يعد مهماً. نعتقد أنه على الرغم من أن هذه الدراسة تعتبر دراسة أولية حول هذا الموضوع، إلا أنها تضع مثلاً للدراسات التي ستساعد في تحديد نسبة الحدوث لداء المقوسات الخلقي بواسطة الفحص على عدد أكبر من السكان في منع النتائج بواسطة التشخيص المبكر.

Objectives: To elucidate the prevalence of congenital toxoplasmosis in Turkey.

Methods: This study was conducted in the Department of Parasitology, Faculty of Medicine, Ege University, Izmir, Turkey during the period of 2006 and 2007. Venous blood before delivery and cord blood during delivery were collected from 138 women, and we observed the presence of immunoglobulin (Ig)G and IgM antibodies by Enzyme Linked Immunosorbant Assay (ELISA), western blotting, and other serologic tests.

Results: The combination of ELISA and western blotting have the greatest sensitivity among the serological techniques used. The results of the cord and venous sera were comparable with no significant difference, except for one sample.

Conclusion: Data obtained showed that early characterization of IgG antibodies synthesized by congenitally infected newborn is important. This preliminary study sets an example to the studies that would help both in determining the incidence of congenital toxoplasmosis by screening larger populations and in preventing sequels by early diagnosis.

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Congenital toxoplasmosis usually occurs by infection of the mother during pregnancy or 6-8 weeks prior to gestation. Congenital toxoplasmosis rate differs worldwide and is estimated to affect 1-10 per 10,000 newborns in Europe.^{1,2} Toxoplasmosis in fetus may end up with severe congenital defects such as hydrocephalus, mental retardation, and retinochoroiditis, which may be present at birth or developed later in life.¹⁻³ The chance of fetal infection was published as 20-40% when non-immune pregnant women were infected. The invasion period is a short lasting one due to the production of protective antibodies, which passes from mother to the fetus. Although the parasite can live in the fetal nerve tissue and retina, it disappears

in other tissues. The period required for the transition of the infection from placenta to fetus is considered related to some factors such as virulence of *Toxoplasma gondii* (*T. gondii*), amount of inoculation, gestational age, and some other factors.¹⁻³ Studies related to the epidemiology of congenital toxoplasmosis in some European countries revealed detailed information. The screening for toxoplasmosis has been carried out in France and Austria as a mandatory part of prenatal care.⁴⁻⁵ Prenatal screening has been carried out in pilot projects in Germany, Finland, and Norway.⁶⁻⁸ However, the prevalence of congenital toxoplasmosis in Turkey is not known. This study was designed to elucidate the prevalence of congenital toxoplasmosis in Turkey.

Methods. Two hundred and seventy-six samples (138 serum of mother blood, 138 serum of cordon blood) were collected from pregnant women who delivered at Ege University Medical Faculty Hospital, Izmir, Turkey. Pregnant women who had known systemic diseases (hypertension, diabetes mellitus, and so forth) were excluded. Western blotting and enzyme-linked immunosorbent Assay (ELISA) were performed on all cord bloods after collection, whereas IgM immunocapture and IgM immunosorbent adsorption assay (ISAGA) were only performed on sera of the suspected patients. The mean age of the study population was 26.3 years (range 16±42).

Antigen preparations were made from tachyzoites of the thyrotropin-releasing hormone (TRH), strain (RH like strain; personal information from Dr. Sibley) of *T. Gondii*.⁹ Tachyzoites were obtained from the peritoneal exudates of mice infected 2 days earlier. These antigens were then routinely used for the serological *Toxoplasma* tests.¹⁰ The study protocol was approved by the local research ethics committee and informed consent was obtained from all participants.

Laboratory analyses. *Western blotting.* Cord blood samples from neonatal and mother blood samples were received serum and confirmed infection by western blot for IgG antibodies according to a previously reported method. Antibodies uniquely produced by newborns and mothers were detected by western blotting.¹¹⁻¹³ Briefly, *T. gondii* tachyzoites (15x10⁶ 95% pure parasites per gel) were separated by electrophores on a 12% sodium dodecyl sulfate-polyacrylamide gel and transferred onto nitrocellulose membranes by standard techniques.^{14,15} The membranes were blocked with 5% low fat dried milk, cut into 0.3 cm strips, incubated with the serum samples diluted 1:200, developed by incubation with peroxidase-anti-human IgG conjugate (1:2,000 dilution) washed with 0.01 M phosphate buffer, pH 7.2, 0.15 M NaCl 0.05%, Tween 20, and treated with

substrate/chromogen solution containing H₂O₂ and 4 chloro-1-naphthol.

Enzyme Linked Immunosorbant Assay. The wells in microtiter plates were sensitized with the toxoplasma antigen overnight at 48°C. The plates were then washed 3 times in 3 min with phosphate buffer solution (PBS) containing 0.05% Tween 20; and 100 µl diluted serum was added to each well and incubated for one hour at 37°C. The plates were washed as before in PBS/Tween 20. Alkaline phosphatase conjugated anti-human IgG or IgM (100 µL each) (Sigma A3187, Sigma A-3437) were added to each well and incubated for one hour at 37°C in the presence of 100 µL substrate.⁹ The enzymatic hydrolysis of substrate was stopped after 30 min by the addition of 100 µl NaOH. Samples with absorbance values greater than 2 or usually 3 times of the absorbance values of negative control were considered positive.⁹

Immunoglobulin M immunocapture. Microtiter plates were coated with 100 µl rabbit anti-human IgM (Dako, Glostrup Denmark) and kept at 48°C overnight, washed 3 times with 0.0005% Triton-X included PBS (pH 7.2). Serum samples (100 µl) diluted in 1:100 were tested in duplicate. The microtiter plates were incubated at 37°C for one hour and washed for 3 times. *Toxoplasma gondii* antigen (100 µl) was applied and incubated at 37°C for one hour, washed, and 100 µl mAb was added per well and incubated at 37°C for one hour. Rabbit anti-mouse immunoglobulins (100 µl) with conjugated alkaline phosphatase (Dako, Glostrup Denmark) were applied and incubated at 37°C for one hour and washed (Eskild). Enzyme substrate [p-nitrophenyl phosphate] (100 µl) were applied and incubated for 30 min before the reaction was stopped. We considered positive those with more than 8 IU. World Health Organization International standard sera (Statens Serum Institut, Denmark) were used as negative and positive controls.^{9,16}

Immunoglobulin M immunosorbent agglutination assay (ISAGA). We used IgM ISAGA (bioMerieux) in this study. Manufacturer suggested that even a low ISAGA index (≥3) might indicate congenital toxoplasmosis.^{9,17}

Statistical Package for Social Sciences (SPSS, version 10.0) software was used to calculate descriptive statistics. Please provide detailed statistical analysis.

Results. In this study, venous blood samples before delivery and cord blood samples during delivery were collected from 138 women. Immunoglobulin G and IgM anti-toxoplasma antibodies were searched in 276 sera using ELISA. Among 138 samples taken from pregnant women, 78 (56.5%) were IgG positive in ELISA tests. Distribution of toxoplasmosis seropositivity with respect to the age is summarized in Figure 1.

Toxoplasmosis seropositivity increased in parallel to the age (Figure 1). Comparisons were made between venous blood sera and cord blood sera; except in one sample there is no significant difference was observed. The ELISA titration values for IgG in both venous and cord sera ranged from 1/16 to 1/1024. Immunoglobulin M titration values were determined in 1/64 for 3 samples and 1/128 for one sample. On these 4 IgM positive we performed ISAGA and immune capture and only the sample, which had 1/128 titration value, was positive for both ISAGA and immune capture. The rest was negative for ISAGA and immune capture.

Western blotting was performed to compare the band profiles of seropositive venous and cord sera. Except the sample that we found positive for both ISAGA and immune capture. Similar band profiles were observed (Figure 2). The appeared band profiles paralleled between the sera of mothers and cord bloods. The detected bands appeared at 105, 80, 60, 50, 48, 38, 35, 30, 25, 22, and 20 kDa molecular masses. No

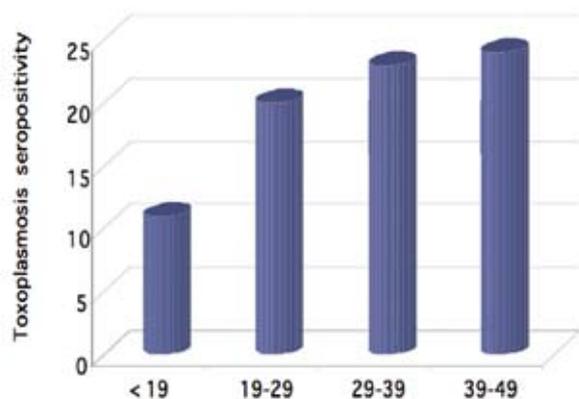


Figure 1 - Distribution of toxoplasmosis seropositivity with respect to the age.

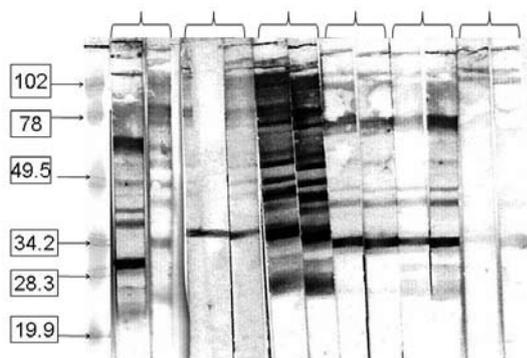


Figure 2 - Examples of the different Western blot patterns obtained amongst the 138 mother/cord blot pairs. Presence of neosynthesized IgG in infected newborn (no. 1). The molecular mass markers in kilodaltons are shown on the left side of each blot.

statistically significant difference was found between ELISA IgG and western blotting ($p > .05$; χ^2 test). The bands appeared at 38, 22, and 20 kDa ranges were the most observed. In this one sample, the ELISA test values in venous and cord sera for IgG were 1/1024 and 1/512, respectively, and for IgM were 1/128 for both. The child who was cord seropositive for toxoplasmosis was followed up for 2 years but no signs of sequelae, either neurological or ophthalmological were found.

Discussion. Although the aim of this study was to investigate the congenital toxoplasmosis, we also discussed the characteristic of the 2 serological tests: ELISA and western blotting. No program has been planned for detection of congenital toxoplasmosis in Turkey and only local studies have been performed. However, many of these local studies focused on toxoplasmosis seropositivity rather than congenital toxoplasmosis. According to these local serological studies, the seroprevalence ranged between 17.3-78%.¹⁸⁻²³ Among 138 samples taken from pregnant women, 78 (56.52 %) were IgG positive in ELISA tests. And in another study, out of 1972 pregnant women, seropositivity for anti-toxoplasma IgG antibody was found in 952 (48.3%).²⁴ Ertug et al²⁵ reported seroprevalence and risk factors for toxoplasma infection among pregnant women in Turkey. They found that seroprevalence was increased with age and with drinking water consumption other than bottled water.^{25,26} We did not look at risk factors for toxoplasma infection among pregnant women in this study. The high seroprevalence of *T. gondii* in Turkey is related to the presence of a great number of stray cats in both rural and urban areas of the country. These animals feed on rodents and birds acquiring infection and shedding millions of oocysts in the environment. The Turkish diet, which consists of large amounts of raw, wild vegetables, and salads that could easily be contaminated with parasite oocysts shed by infected cats also plays an important role. Furthermore, undercooked lamb, sheep, goat, and meat are the well-documented sources of *T. gondii* and are highly consumed.^{1,26}

In a study in Izmir, the sera of 300 mothers were tested with ELISA and 45.3% for anti-toxoplasma IgG and 1.7% for anti-toxoplasma IgM were found to be positive. The rate of prenatal toxoplasmosis was determined in 3 (1%) of the 300 cord blood samples tested for IgM antibodies.²⁷ In the present study, we looked for seropositivity in congenital toxoplasmosis in Turkey, which makes this study unique. However, we have to make a note that the limitation of this study is that it only represents the cases within a hospital but not the cases from various hospitals. In general, serological screening was performed with ELISA because it is a

sensitive and economic technique. All positive samples determined by ELISA were then confirmed by ISAGA.¹³ In addition; in some studies western blotting was used as an additional informative approach. Previous studies reported that western blotting method had a place in the diagnosis of congenital toxoplasmosis and this technique was extremely important in distinguishing the antibodies synthesized by fetus or newborn and by fetus or newborn and the mother. By using the immuno-blotting technique, the antibodies against various antigens of *T. gondii* can be searched and these antigens can be defined.^{12,28} In this study, all of these techniques were used.

The western blotting profiles observed in this study were comparable with another study. The most evident band in western blotting IgG was reported to be 35 kDa in chronic infection. Other bands varying between 150, 115, 105, 60, 35, 50-58, 30, 27-29, and 20-22 kDa were also detected.²⁹ Development of antibody response against antigens with molecular weight of 86, 63, 55, 42 kDa was different in the mother and IgG antibodies detected in the baby were considered not as mother originated, but as synthesized by the infant. In cord bloods, those were included in the study; none of these bands were found. It was noted that the number and the size of the band could be in great variability from patient to patient and this was the result of the changes in the individual reactions of the patient or different subspecies forming the infection.³⁰ When the literature was examined, it was seen that antigens of 21, 26, 28.5, 39, 57, 69, 97, 108 kDa molecular masses were *T. gondii* E/S antigens, those of 6, 22, 30, 35, 43 kDa molecular masses were surface antigens, and those of 23, 28, 55-60, 58, 88, 98 kDa molecular masses were intracellular antigens.³¹⁻³³ Existence of surface antigens of various molecular weights, those reported to be detected specific to *T. gondii*, was also frequently seen in the cases found positively during the assessments by western blotting in our study. It was reported that bands appeared against more than 20 antigens ranging from 4-150 kDa with western blotting using IgG antibody the sera of cases with acute toxoplasmosis.^{31,32} In these cases, the majority of the bands defined by western blotting IgM were also seen in western blotting IgG. In the cases of acute toxoplasmosis, the strongest IgG response was detected at the antigenic band of 35 kDa and the weakest response, in contrast to western blotting IgM, occurred against the antigen between 4-6 kDa.³¹⁻³³ In the present study, this was the most frequently seen band after the one having a molecular mass of 38 kDa with western blotting. Antibody response by western blotting was observed in 78 of totally 138 venous cord bloods collected. Sera of subjects infected with *T. gondii* were analyzed by western blotting both IgM and IgG

antibodies were reported to recognize antigens of 6, 22 and 32 kDa.^{13,31} In this study, we observed the existence of antibodies produced against antigens of 22 kDa molecular weight in 43 of 72 cases found positively by western blotting.

Detection of IgG and particularly IgM or IgA synthesized by newborn at birth is important in early diagnosis of congenital toxoplasmosis. With early diagnosis, severe disabilities are prevented by the onset of specific treatment in infected infants. It is noted that complementary techniques are required in the serological diagnosis of congenital toxoplasmosis after birth. There are inadequate techniques in detecting the titration of IgM synthesized by fetus. Still, immune capture tests have been reported to be highly sensitive in detecting IgM.¹³ Congenital toxoplasmosis in newborns can be diagnosed late by IgG titers usually detected in months 6-9. In one study, congenital toxoplasmosis was even diagnosed by analyzing the urine samples of 4 newborns with PCR.³⁴ In the present study, patterns of Ig G and/or anti-Toxoplasma antibodies of the mother and the newborn were obtained. Some authors demonstrated characteristically specific IgG bands in cerebrospinal fluid samples and ocular fluid of congenitally infected newborns with western blotting.³⁵ They observed that performing western blotting was easier than ELISA, but the bands should be read carefully. It was reported that specificity of western blotting, particularly in determining IgM seropositivity in cord sera, was higher than immune capture tests, but not the sensitivity.¹² In the first 3 months, while other tests are negative, western blotting should be repeated. Numerous anti IgG bands were defined between 5 kDa-117 kDa. The differences among these bands were related to their antigenic characteristics. In addition, as defined previously, some of IgG and IgM epitopes could be used collectively. Similarly, it was reported that IgA pattern was in common with IgG and IgM patterns, but sensitivity of IgA was weaker than other immune capture tests.¹³ During our study, we statistically evaluated the results obtained by ELISA and western blotting methods, and facilitated the diagnosis of toxoplasmosis, and found no significant difference between the results obtained with both tests (χ^2 , $p>0.05$).

In conclusion, the data obtained showed that early characterization of Ig antibodies synthesized by congenitally infected newborn is important. We think although that this study is a preliminary study on this topic it sets an example to the studies that would help both in determining the incidence of congenital toxoplasmosis by screening larger populations and in preventing sequels by early diagnosis.

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