Congenital Toxoplasmosis: Evaluation of Molecular and Serological Methods for Achieving Economic and Early Diagnosis Among Egyptian Preterm Infants

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Summary

Background: Early diagnosis of congenital toxoplasmosis (CT) is difficult when specific immunoglobulin M (IgM) antibodies are absent, or if persist for months, in the newborn infant’s blood. Objectives: To study the risk factors of neonatal toxoplasmosis and to compare different immunologic profiles (Toxoplasma-specific IgM, IgA antibodies and the avidity of IgG antibodies) with polymerase chain reaction (PCR) for reaching economic and early postnatal diagnosis.

Materials and Methods: We prospectively studied 80 preterm neonates, recruited from neonatal intensive care units (NICUs) of Cairo University hospitals. Whose gestational age ≤34 weeks with (n = 60) or without (n = 20) CT risk. Serum samples for specific IgA, IgM antibodies and avidity of IgG toxoplasma antibodies were measured by ELISA then compared to PCR. Results: Of the 60 studied cases, 16 (26.7%) were positive for toxoplasmosis by PCR, of which 15 (25%) had low avidity of IgG antibodies (positive), 14 (23.3%) were positive for IgA and 10 (16.7%) were positive for IgM, with sensitivity for avidity of IgG, IgA and IgM: 93.2%, 87.5% and 62.5%, respectively. Conclusion: Determination of avidity of IgG toxoplasma antibodies and/or serological detection of specific IgA for toxoplasmosis offer, simple tests for diagnosis of congenital toxoplasmosis with (better sensitivity) than IgM.

Key words: Congenital Toxoplasmosis (CT), IgM, IgA, avidity of IgG test, polymerase chain reaction (PCR).

Introduction

Toxoplasma gondii (T. gondii) infection is a growing worldwide health problem. Regarding seropositivity, the prevalence of T. gondii in Egypt between August 2007 and October 2008 was 57.6% in rural area vs. 46.5% in urban residences [1]. The rates of mortality or morbidity sequelae in congenitally infected neonates range between 1 per 10 000 and 80 per 10 000 births [2, 3]. In case of preconception infection, the developed protective immunity prevents fetal transmission. However, immunocompromised women may transmit the parasite vertically [4]. Fetal transmission depends on occurrence of maternal parasitemia, cellular immunity and stage of placental development [5]. Placental colonization with T. gondii is a potential source of fetal infection long after maternal parasitemia has subsided [6].

The clinical spectrum of congenital infection ranges from apparent developmental alterations at birth, with high perinatal morbidity and mortality (microcephaly, intracranie growth retardation and hydrocephaly), to subclinical infection, with a risk of developing retinochoroiditis and other complications [7, 8]. Ocular sequelae may be prevented by early postnatal diagnosis and treatment with pyrimethamine and sulfonamides [5, 9].

Current methods of immunoglobulin M (IgM) screening frequently fail to establish early diagnosis. Growing interest in the use of specific anti-Toxoplasma IgA (no natural interference for IgA; earlier formation and shorter persistence in serum) appears useful as an adjunctive method for identifying recently acquired infection [10].

Avidity of toxoplasma IgG antibodies is the binding force of the antibody (serum specimen) with the
corresponding antigen, which is initially low after primary antigenic challenge and increases during subsequent weeks and months by antigen-driven B-cell selection (maturation of antibody affinity). The concept in this test depends on protein-denaturing reagents, including urea that is used to dissociate the antibody–antigen complex based on the weak affinity early in infection which becomes stronger late (in chronic infection). High-avidity test excludes the recent (acute or congenital) infection while low-avidity test states recent infection [11].

Using enzyme-linked immunosorbent assay (ELISA) quantification of both specific IgM and IgA antibodies and the avidity test of IgG toxoplasma antibodies may be of value to identify suspected cases at birth [12].

In the study done by Chiabchalard et al. [13], polymerase chain reaction (PCR) could detect as few as 0.25 parasites per 100 000 human leukocytes, which is very sensitive.

We hypothesize that comparative approaches between the immunologic profiles and PCR may help much in early diagnosis of missing congenital Toxoplasmosis (CT) cases.

Study Design

Subjects

We confined analysis to preterm neonates of neonatal intensive care units (NICUs) of Cairo University (Kasr Al Aini and Abou-El Riche). Approval by the University Ethical Committee Board at and parental consents were obtained.

Maternal questionnaires (regarding mothers’ age, residence area and antenatal habits together with symptoms suggestive of Toxoplasma infection during pregnancy) were prepared. Neonates with consanguineous parents were excluded from the study.

Eighty preterm neonates ≤34 weeks gestation were prospectively enrolled from July 2008 till October 2009. Sixty suspected cases were selected according to the presence of one or more of the following: prematurity or signs of intrauterine growth retardation, jaundice or elevated liver enzymes, hepatosplenomegaly (HSM), hydrocephalus, microcephaly or seizures, cardio respiratory problems, sepsis, chorioretinitis or intracranial calcifications. Twenty healthy preterm neonates with birth weight ≥1800 g were recruited from obstetric hospital to serve as control group. After routine care and stabilization, they were enrolled in the study followed by discharge with their healthy mothers. Their follow-up in the outpatient clinic revealed no serious problems apart from physiological jaundice in five cases, where three infants only required phototherapy (≤3 days).

All neonates were submitted to full antenatal, natal and postnatal history taking including mode of delivery, gender, birth weight and assessed gestational age [14]. Neonatal sepsis was diagnosed on clinical basis and laboratory data (positive blood culture, positive or CRP value >10 mg l \(^{-1}\) or immature: total neutrophil (I/T) ratio >0.25. Ophthalmologic assessment, chest X-rays, echocardiogram, cranial ultrasound and/or computerized tomography were performed when needed and results were recorded.

Methods

Blood sampling and analysis

Peripheral blood (3 ml) was collected from the neonates in the first 7 days of life. Each sample was divided into two tubes, one preserved on EDETA for PCR and the other sample was centrifuged, where serum was collected for IgM, IgA and IgG avidity test and both samples were kept at –20°C. Other laboratory tests included complete blood count, C-reactive protein (CRP), blood culture and liver function tests were done.

For molecular test, two positive serum samples (based on positive IgM and confirmed with positive PCR) were used as positive control.

PCR is defined as the reference standard for congenital toxoplasmosis status. PCR was compared with the presence of specific IgM, IgA antibodies and low-IgG avidity test by ELISA.

IgM detection. The Bio-Quant Inc. (BQ) Toxo (IgM) ELISA kit was used. The test is based on the sandwich enzyme immunoassay principle [15]. The absorbance of the calibrator was measured and the cut-off value was calculated. The optic density (OD) of each sample and controls was measured. For antibody index interpretation: <0.9 was interpreted as negative, 0.9–1.1 was interpreted as equivocal and >1.1 was indicative of Toxoplasma infection.

IgA detection. ImmunoLISA TM Toxo IgA ELISA kit was used. The test is based on the capture enzyme immunoassay principle for detection of IgA class antibodies to Toxoplasma in human serum [16]. For calculation of the results, the cut-off value was calculated: OD 450-nm value lower than the cut-off were classified negative, OD 450-nm value within the cut-off +20% were considered in a gray-zone and OD 450-nm value higher than the upper limit of the great-zone were classified positive.

IgG avidity determination. NovaLisa TM T. gondii IgG avidity test is an enzyme immunoassay for the avidity determination of IgG-class antibodies to T. gondii in human serum [17]. Interpretation of results: avidity (%) >40 (toxoplasmosis antibody with high avidity) means past infection, while avidity (%) ≤40 (toxoplasmosis antibody with low avidity) means acute or recent infection.
Molecular assay PCR. Processing of DNA by PCR involves three main steps: DNA extraction, DNA amplification and detection of amplified product by agarose gel electrophoresis. DNA isolation was done. Two oligonucleotide primers: (GGA ACT GCA TCC GTT CAT GAG) and (TCT TTA AAG CGT TCG TGG TC) were designed in order to amplify T. gondii-B1 gene. The visual inspection of amplified products was done after electrophoresis separation on a 2% agarose gel. After migration, DNA bands in the gel could be visualized after exposure to the UV transillumination apparatus compared to the positive Toxoplasma DNA control (193 bp) [18].

Statistical methods
Data were coded and entered using the statistical package SPSS version 15. Data were summarized using mean and standard deviation (SD) for quantitative variables and number and percent for qualitative variables. Comparison between groups was done using chi-square test for qualitative variables and independent sample t-test for normally distributed quantitative variables, while non-parametrical Mann–Whitney test was used for quantitative variables which are not normally distributed. p-values <0.05 were considered statistically significant.

Validity of serological tests was done (compared to PCR) by calculating sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and total accuracy [19].

Results
Out of the studied 80 preterm neonates, 60 were suspected for toxoplasmosis as cases group and 20 healthy preterm infants served as control group. Both were investigated for T. gondii infection using serological and molecular studies and the results were interpreted.

The 60 suspected cases were 33 (55%) females and 27 (45%) males with mean gestational age 31.9 ± 2.2 weeks. Mean birth weight was 1590.3 ± 298.5 g, with mean length 40.9 ± 3.1 cm and mean head circumference 29.9 ± 2.7 cm. Forty-four infants (73.3%) were born by normal vaginal delivery. Of the sixty studied cases, 48 (80%) were discharged and 12 (20%) died due to severe neonatal sepsis, pneumonia or heart failure.

The control group (n = 20) were 12 (60%) females and 8 (40%) males with mean gestational age 32.1 ± 1.8 weeks. Mean birth weight was 1800 ± 259.6 g with mean length 42.1 ± 3.5 cm and mean head circumference 29.7 ± 3.5 cm. Fifteen neonates (75%) were born by normal vaginal delivery.

Relation between such risk factors and neonatal toxoplasmosis is demonstrated in Table 1. Clinical and laboratory data of cases together with PCR results are summarized in Table 2 and Table 3.

Of the 60 studied cases, 16 (26.7%) were positive for toxoplasmosis by PCR, of which 14 (23.3%) were positive for IgA, 10 (16.7%) were positive for IgM and 15 (25%) showed low-avidity test for IgG. This is demonstrated in Fig. 1. Of the control group, 15 (60%) of the infants were positive for IgG, 9 (45%) for IgM and 7 (35%) for IgA.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Relation between different maternal risk factors for toxoplasma infection and PCR Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor</td>
<td>Negative control group n = 20</td>
</tr>
<tr>
<td>Residence area (Rural)</td>
<td>Percentage of risk factor in mothers (%)</td>
</tr>
<tr>
<td>Educational level (Non-educated)</td>
<td>10 (50)</td>
</tr>
<tr>
<td>Contact with cats</td>
<td>12 (60)</td>
</tr>
<tr>
<td>Contact with soil (Gardening or agriculture)</td>
<td>15 (75)</td>
</tr>
<tr>
<td>No washing of hands before meals</td>
<td>18 (90)</td>
</tr>
<tr>
<td>Eating raw or processed meat</td>
<td>5 (25)</td>
</tr>
<tr>
<td>Symptoms suggestive of Toxoplasma infection during pregnancy*</td>
<td>0 (0)</td>
</tr>
<tr>
<td>History of immune weakness during pregnancy</td>
<td>0 (0)</td>
</tr>
<tr>
<td>History of abortions or congenital malformation of previous baby</td>
<td>4 (20)</td>
</tr>
</tbody>
</table>

NS, non-significant difference.
*Fever, headache, painless swollen lymph glands, muscle aches, fatigue and possibly sore throat or rash.
five neonates (25%) had physiological jaundice without any further clinical or lab evidence of complications.

Serological results of neonates (IgM, IgA and IgG avidity test) were compared to PCR results since definition of positivity was based on positivity of PCR. Table 4 and Fig. 2 reveal that IgG avidity has the highest validity followed by IgA. IgM was the least sensitive as compared to IgG avidity test and IgA. Both IgG avidity, IgA and IgM showed high specificity and PPV (100%).

Discussion

Advances in early diagnosis coupled with early and prolonged treatment during the first year of life appeared to modify the course of CT. Infected infants may have clinical manifestations, but still they can be asymptomatic at birth, with later serious ocular and CNS sequela [20].

Fourteen neonates to 34 rural mothers (41.2%) were positive, with \( p \)-value (0.031). This may be related to bad sanitary conditions, wide spread of stray cats or due to low educational level in rural areas. This agrees with Foulon et al. [20] and Spalding et al. [21] who reported higher risk of toxoplasmosis in subjects living in rural (73.6%) than those living in urban areas (42.2%). Epidemiologic data reported by others supported the present results of higher prevalence of this zoonotic infection in rural areas [22]. Many authors have emphasized the role of cats as well as the antenatal ingestion of poorly cooked meat in transmission of toxoplasmosis [23, 24].

The current study shows high association between intracranial calcifications, chorioretinitis or sclerema and congenital toxoplasmosis (in 100% of studied cases). Sepsis and HSM are also associated with congenital toxoplasmosis in (67%) and (53%) of cases, respectively. A result agreeing with Lebas et al. [25] who recommended that early serologic tests and ophthalmologic investigations should continue in co-operation with pediatricians to reduce long-term complications.

On the other hand Cneude et al. [26] reported association between neonatal HSM, pneumonitis and sepsis with underlying undiagnosed toxoplasmosis.

According to the findings in the current study, all tests were able to exclude all normal subjects (control group) but showed differences in diagnosis of the group of suspected cases. There is overall high positivity regarding molecular diagnosis, 16 neonates (26.7%) were positive. Agreement of PCR results and the detection of IgM antibodies against *Toxoplasma* is found in 10 (16.7%) of cases by IgM, while IgA was positive in 14 (23.3%). Regarding IgG avidity test, 15 (25%) of cases are ruled to have acute infection being of low-avidity value.

| Table 3 |
| Relation between different studied variables and PCR findings in neonatal toxoplasmosis |

<table>
<thead>
<tr>
<th>Variable</th>
<th>PCR positive ((n = 16))</th>
<th>PCR negative ((n = 44))</th>
<th>(p)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational age (weeks)</td>
<td>32.25 ± 1.5</td>
<td>31.8 ± 2.4</td>
<td>0.499</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>1572.5 ± 222.7</td>
<td>1596.8 ± 323.8</td>
<td>0.744</td>
</tr>
<tr>
<td>Length (cm)</td>
<td>40.4 ± 2.7</td>
<td>41.1 ± 3.3</td>
<td>0.439</td>
</tr>
<tr>
<td>Head circumference (cm)</td>
<td>29.9 ± 3.2</td>
<td>29.8 ± 2.5</td>
<td>0.893</td>
</tr>
<tr>
<td>HB mg%</td>
<td>10.6 ± 1.9</td>
<td>11.6 ± 2.6</td>
<td>0.156</td>
</tr>
<tr>
<td>WBCs (\times 10^3\ mm^{-3})</td>
<td>8.050 ± 4.9</td>
<td>7.850 ± 3.3</td>
<td>0.401</td>
</tr>
<tr>
<td>Platelets (\times 10^3\ mm^{-3})</td>
<td>129.375 ± 71.4</td>
<td>150.681 ± 83.8</td>
<td>0.481</td>
</tr>
<tr>
<td>SGOT units</td>
<td>39.1 ± 11.9</td>
<td>38.3 ± 9.5</td>
<td>0.810</td>
</tr>
<tr>
<td>SGPT units</td>
<td>34.0 ± 6.4</td>
<td>31.0 ± 4.9</td>
<td>0.063</td>
</tr>
<tr>
<td>Total bilirubin mg dl(^{-1})</td>
<td>14.3 ± 2.7</td>
<td>14.2 ± 2.0</td>
<td>0.947</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD.
Regarding sensitivity of IgM, IgA and IgG avidity, IgA is more sensitive (87.5%) compared to IgM (62.5%), while IgG avidity test showed the highest sensitivity (93.2%). In agreement with our results, Spalding et al. [21] pointed out that the detection of specific anti-Toxoplasma IgA antibodies instead of anti-Toxoplasma IgM antibodies for the early diagnosis of acute congenital Toxoplasma infection has...
been well established with sensitivity and specificity 90.0% and 100%, respectively. Meanwhile, our results regarding IgM are validated by Candofi et al. [27] who noted that specific IgM antibodies to Toxoplasma are often poorly informative as marker of acute infection, with sensitivity 67% which mandated a complementary diagnostic technique on the first sample. Moreover, Kodym et al. [28] demonstrated that positive specific IgA is more indicative for acute T. gondii infection than specific IgM as it disappears earlier (between 3 and 9 months) while IgM remains at high levels for several months (up to 1 year), rendering inadequate sero-diagnosis of acute toxoplasmosis.

In the present study, the IgG avidity test that showed high sensitivity 93.2% with total accuracy 98.3% provides an additional confirmatory test. Thus low IgG avidity showed higher validity than IgM and IgA being low in 15 cases, from which 10 cases only showed positive specific Toxoplasma IgM and 14 showed positive specific Toxoplasma IgA. This is supported by Chernecky and Berger [29] who reported that negative Toxoplasma IgM does not rule out recently acquired infection and that IgG avidity test enables to differentiate recently acquired from past infection. Low-avidity test has been utilized as an additional tool for decision making in management of equivocal results [11].

Despite the IgG avidity test’s usefulness in addition to the discriminatory power of serology in distinguishing recently acquired from chronic infection, yet its results may be misinterpreted as acute infection if used alone. Such discrepancy is attributed to a reflection of the various windows of time (different from one patient to another) at which different tests’ results evolve from acute to chronic [30].

There is a higher reliability of diagnosing specific IgM or IgA with an increase in overall sensitivity when immunoglobulins are jointly assessed. This improved the diagnostic yield to 89% relative to IgM or IgA alone which were 70.3% and 86%, respectively [31].

Not all congenitally infected infants produce detectable levels of specific IgA or IgM. Therefore, measurement of two of the following: IgG avidity, specific IgM or specific IgA seems to be a recommendable approach [32].

The superiority of PCR sensitivity in diagnosis of congenital toxoplasmosis refers to its ability in detection of the parasite DNA (even if single) in blood.

According to Romand et al. [33] and Bessières et al. [34] PCR had the highest levels of sensitivity and specificity in the diagnosis of (prenatal and postnatal) toxoplasmosis in comparison to serological tests.

Other researchers recorded that all patients with cerebral toxoplasmosis presented positive PCR results (sensitivity, 100%) [35]. These findings emphasize the clinical utility of PCR in the diagnosis of cerebral toxoplasmosis and for its usage as a reference standard for comparison with other tests.

Besides, PCR does not depend on the immune response of the patient as the serological tests do [30]. Meanwhile, in our study, serological detection of IgA and IgG avidity test carries the great value being less expensive and technically uncomplicated.

The current study could have been improved if carried out on a wider scale (i.e. multi-center setting) to allow more chances for better screening of larger population. Our study faced two limitations; first, is the absence of antenatal serological tests which were impractical for the rural residence and second is the small sample size that did not allow different (slightly less) specificity of tests.

Conclusion and Recommendations

Neonatal screening for toxoplasmosis is very important to reduce complications. Suspected cases with ocular lesions or neonatal sepsis must be investigated. IgG avidity test could permit an efficient, easy and non-expensive test for congenital toxoplasmosis diagnosis, however, better to be accompanied by serological detection of IgA if possible on financial basis. PCR is still the most accurate diagnostic technique; however, its high cost and complexity lead to limited value as a screening test.

References


