Detection of Immunoglobulin M Antibodies Specific for *Toxoplasma gondii* with Increased Selectivity for Recently Acquired Infections

Ravi Kaul,* Peilin Chen, and Steven R. Binder

Clinical Diagnostics Group, Bio-Rad Laboratories, Hercules, California

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*Toxoplasma gondii* infections can cause serious complications in pregnant women, leading to miscarriage, stillbirth, and birth defects. Definitive diagnosis of *T. gondii* acute infection is therefore critical for the clinical management of a mother and her fetus. Positive immunoglobulin M (IgM) results are not sufficient as evidence of recent infection, as these antibodies are often present for many months. Further, IgG avidity and differential agglutination tests, two tests used by reference laboratories to distinguish between recent and past infections, are not always in agreement, and both methods yield a significant number of indeterminate results. We report the development of a new toxoplasma IgM immunoassay that is performed by using a bead-based immunoassay on an automated analyzer (BioPlex 2200). Initial validation included 204 samples from pregnant women and 198 samples from asymptomatic healthy adults. An overall specificity of 99% was observed. Further, 100% sensitivity for acute infections was observed for 10 well-characterized seroconversion panels. We then examined 50 samples from pregnant women, all of which were IgM positive by ELISA, which had been fully evaluated in a reference laboratory. Of the 50 samples, 34 (68%) tested positive in the BioPlex 2200 toxoplasma IgM assay, of which 32 of 34 (94%) exhibited an acute or equivocal pattern by differential agglutination. Of the 16 negative samples, 15 (94%) showed high-IgG-avidity antibodies. Collectively, these results suggest that this new toxoplasma assay shows a preferential response to IgM antibodies produced by recent infections, reducing the number of positive results for pregnant women that will require extensive additional clinical evaluation.

*Toxoplasma gondii* is an obligate intracellular protozoan responsible for common parasitic infections throughout the world. It affects a wide range of hosts, including humans, domestic mammals, and birds, with members of the cat family being the only known hosts for the sexual stages of *T. gondii* infection. In general, *T. gondii* infections are asymptomatic and self-limiting, especially among healthy immunocompetent hosts; however, the infection may cause severe complications in pregnant women and immunocompromised patients (6). Fetal infection can develop only when a woman with no immunity becomes infected with *T. gondii* during pregnancy or up to 8 weeks before conceiving. Fetal toxoplasmosis, particularly in early pregnancy, can cause miscarriage, stillbirth, and birth defects. Infected babies may not develop any disease, or they may experience serious damage to the brain and eyes. The detection of recently acquired infection in pregnant women is therefore critical for clinical management of the mother and her fetus (19, 20, 23, 25). PCR amplification of *T. gondii* DNA in blood cells, body fluids, and tissues has been used successfully for the diagnosis of congenital toxoplasmosis; however, its reliability with amniotic fluid samples before 18 weeks of gestational age is unknown (5, 21). Furthermore, PCR amplification is not very helpful in predicting the time of infection, because the clearance of DNA from body fluids and tissue samples is not well established.

The detection of *T. gondii*-specific immunoglobulin M (IgM) antibodies is the most common method used to determine the point during pregnancy when the infection occurred. In the United States, the incidence of acute toxoplasma infection during pregnancy has been estimated to be approximately 0.2 to 1.0%, although in about 90% of cases the disease goes unrecognized (25). In some countries, the seroprevalence of IgM antibodies has been reported to be as high as 2.4% (23). Identifying the time of primary infection is crucial for the clinical management of pregnant women because of an inverse correlation between the severity of toxoplasmosis for the fetus and the gestational age (14, 25). However, variability among in vitro commercial kits prompted the United States Food and Drug Administration (FDA) to reexamine the performance of these kits in 1997 (24). The clinical specificity of these kits varied between 77 and 99% compared to the reference IgM enzyme-linked immunosorbent assay (ELISA) test (17). For this reason, the FDA currently requires additional evaluation of preliminary positive IgM results by a reference laboratory.

Repeat testing of IgM is not sufficient to confirm acute infection because IgM antibodies may persist for months or even years after acute infection (1, 4, 15). In an evaluation of IgM-positive sera from pregnant women, submitted by clinical laboratories using FDA-cleared tests, 60% of the sera were determined to be consistent with chronic (nonacute) infection when tested by the U.S. reference laboratory for *T. gondii* IgM antibodies at the Research Institute, Palo Alto Medical Foundation (PAMF), Palo Alto, Calif. (13). This reference laboratory uses a toxoplasma serum profile that includes a double sandwich IgM ELISA (17), a differential agglutination test (AC/HS test) (2), and the Sabin-Feldman dye test (22) in combination with IgG avidity results (8, 11, 12, 16) to distinguish between recently acquired and distant infections. A positive IgM and IgG test with a high IgG avidity test during the first trimester rules out acute infection in women, but the
presence of low-avidity IgG antibody does not rule out a non-acute infection (15). In this situation, the results from the additional methods are carefully considered. Because no avidity or differential agglutination test has been cleared by the FDA, such tests are performed only at reference laboratories and require carefully trained personnel for proper reading and interpretation of results. Outside of the U.S., a few avidity tests are commercially distributed.

This report describes the development of a new toxoplasma IgM immunoassay that utilizes the Bio-Rad BioPlex 2200 immunomassay analyzer, a fully automated platform with multiplex capabilities (10). The data demonstrate that the conditions employed for the immunoassay yielded a differential response to T. gondii-specific IgM antibodies, with reduced sensitivity to IgM from samples with high-avidity IgG antibodies. This selective response may reduce the number of IgM-positive specimens from women with nonacute infection, reducing the number of patients requiring clinical management.

MATERIALS AND METHODS

T. gondii antigen. The T. gondii antigenic extract from Viral Antigens (Memphis, Tenn.) was reextracted with high-sodium carbonate buffer (1 M sodium chloride in 150 mM sodium carbonate buffer, pH 9.0) for 30 min at room temperature; the purified cell extract was suspended in 10 mM phosphate-buffered saline for subsequent use in the BioPlex 2200 IgM immunomassay. Western blot analyses that were performed on unprocessed and partially purified toxoplasma extracts showed similar antigenic profiles, supporting the observation that antigenic integrity is maintained during the purification process (data not shown).

Serum specimens. Sera from 402 subjects procured from two blood banks were used in this study. The samples included two populations: asymptomatic healthy donors born between the years 1933 and 1994 (n = 198) and pregnant women (n = 204). Of the serum samples from pregnant women, 100 samples were drawn from women in their first trimester, 100 samples were drawn from women in the second trimester, and 4 samples were drawn from women in the third trimester. In addition, nonconsecutive, well-characterized IgM-positive samples from 25 pregnant women after <16 weeks of gestation and another 25 samples from women after >16 weeks of gestation were supplied by the PAMEF. Also, 10 toxoplasma seroconversion panels that were provided by the Hospital La Timone, Marseilles, France, were included in this study. Each seroconversion panel represented three or more sequential serum samples from subjects who were initially IgG and IgM negative for toxoplasma antibodies and then seroconverted in response to acute infection.

Reference assays. The results from the BioPlex 2200 multianalyte immunomassay analyzer were compared to those obtained with several commercially available kits. For specificity, samples were analyzed with the PlateliaToxo IgM (Bio-Rad Laboratories Diagnostic Group, Redmond, Wash.). For seroconversion, samples were analyzed by using bioMerieux (Hazelwood, Mo.) avidity VIDAS and ISAGA toxoplasma IgM kits and the Roche Cobas Core toxoplasma IgG kit. Finally, toxoplasma serum profiles were generated at the PAMEF by using the BioMerieux toxoplasma IgG avidity test (16) as well as the in-house double sandwich IgM ELISA (17), the differential agglutination (AC/HS) test (2), and the Sabin-Feldman dye test (22). Six samples that tested toxoplasma IgM positive by the Platelia and BioPlex 2200 assays were reevaluated at the PAMEF by using their in-house IgM ELISA, AC/HS test, and IgG avidity test.

BioPlex 2200 immunomassay analyzer. The BioPlex 2200 (Bio-Rad Laboratories Diagnostic Group, Hercules, Calif.) is a fully automated, floor-standing random access platform with multiplex capabilities. The instrument combines the Luminex (Austin, Tex.) multianalyte profiling (Lab-MAP) technology (3) with unique antigen-coated fluoromagnetic bead chemistry and versatile software.

The bead reagent was prepared via a three-step procedure involving (i) chemical activation of carboxyl groups on 8-μm-diameter fluoromagnetic polystyrene beads, (ii) covalent coupling of toxoplasma antigen, and (iii) blocking of the unreacted sites with 2% bovine serum albumin (BSA). Essentially, the carboxylic acid groups on the surfaces of fluoromagnetic beads were activated in the presence of N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide and N-hydroxysuccinimide to form activated esters. These esters react with amino groups on purified toxoplasma antigen, yielding stable amide bonds. Following coupling, beads were blocked with 10 mM phosphate buffer, pH 7.4, containing 150 mM sodium chloride and 2% BSA for 2 h at room temperature. Blocked beads were washed three times with bead storage buffer (50 mM Tris-HCl, pH 8.1, 150 mM sodium chloride, and 2% BSA) and stored at 4°C. The amount of toxoplasma antigen coated on beads was titrated for optimal performance.

Buffered solution (100 μl) containing the antigen-coupled fluoromagnetic beads was mixed with 5 μl of serum sample and 100 μl of IgM sample treatment buffer, which contained goat anti-human IgM. The mixture was incubated for 20 min at 37°C, followed by two washes. Subsequently, beads were incubated with 50 μl of an R-phycocerythrin-conjugated goat anti-human IgM reagent (Jackson ImmunoResearch, West Grove, Pa.) for 10 min at 37°C, washed twice, and aspirated into the Luminex detector module. The median relative fluorescent intensity of the beads were measured and recorded. The median relative fluorescent intensity values were converted into an antibody index (AI) by using a 5-point calibration curve created by serially diluting pooled high-titer samples with a pool of toxoplasma-negative blood bank samples. Cutoffs for the BioPlex 2200 assay were adjusted to maximize both assay sensitivity and specificity against the Platelia kit. All samples with an AI value of ≥1.0 were considered positive, while samples with an AI value of <1.0 were treated as negative. Each test includes three internal quality control bead controls designed to monitor detector stability, specimen and sample integrity, and the presence of nonspecific binding that could in accurate serology IgM assays.

RESULTS

Seroconversion panels serve as valuable tools for evaluating the clinical sensitivity of an immunomassay, since the positive samples are known to result from an acute infection. A total of 44 serum samples derived from 10 seroconversion panels were tested to evaluate the performance of the BioPlex 2200 toxoplasma IgM assay. Table 1 compares the BioPlex 2200 toxoplasma IgM test results with bioMerieux VIDAS toxoplasma IgM and bioMerieux ISAGA toxoplasma IgM assay results and the Roche Cobas Core toxoplasma IgG results provided by the Hospital La Timone. The IgG levels were measured to document that each patient was initially seronegative. One hundred percent agreement between the bioMerieux Vidas and BioPlex 2200 toxoplasma IgM test results was observed for all 10 seroconversion panels. One serum sample with an equivocal AI value of 0.6 by the bioMerieux Vidas toxoplasma IgM test was scored as positive. None of the samples reverted to IgG seronegative status, even though the follow-up samples were drawn long after seroconversion (median, 62 days; range, 22 to 247 days). For two of the seroconversion panels, IgM antibodies were detected before IgG antibodies, which accounted for the three additional negative IgG results.

The specificity of the BioPlex 2200 toxoplasma IgM immunoassay was evaluated for 402 retrospectively collected samples. Table 2 lists the serological test results for these samples. None of the 198 asymptomatic healthy donors tested toxoplasma IgM positive by either the BioPlex 2200 or the Platelia assay. In comparison, a toxoplasma IgM positivity rate of 1.5%
TABLE 2. Comparison of the specificity of the Platelia and BioPlex 2200 toxoplasma IgM test results for 402 serum samples

<table>
<thead>
<tr>
<th>Group</th>
<th>Platelia toxoplasma IgM</th>
<th>BioPlex 2200 toxoplasma IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Healthy donors</td>
<td>198</td>
<td>0</td>
</tr>
<tr>
<td>Pregnant women</td>
<td>204</td>
<td>3*</td>
</tr>
<tr>
<td>Total (% positive)</td>
<td>402</td>
<td>3 (0.7%)</td>
</tr>
</tbody>
</table>

*While the number of positive samples identified by the Platelia and BioPlex 2200 toxoplasma IgM assays among pregnant women was the same, the two assays identified different samples as positive. All of the positive results had signals that were less than 1.5 times the cutoff. The reference laboratory results for these six positive samples were negative, and hence these samples were designated as false positives.

(3 of 204) was observed among pregnant-donor samples when tested by either the Platelia or the BioPlex 2200 immunoassay. The three samples that tested positive by the Platelia assay were negative by the BioPlex 2200 assay, and the three samples that tested positive by the BioPlex 2200 assay were negative in the Platelia assay. Repeat tests on these six discrepant samples did not alter the results. Subsequently, these six samples were reevaluated at the reference laboratory for toxoplasma IgM. AC/HS differential agglutination, and IgG avidity. All six samples were negative for toxoplasma IgM, with a nonacute AC/HS pattern. Furthermore, two of six samples showed high-avidity IgG antibodies.

The performance of the BioPlex 2200 toxoplasma IgM assay for pregnant women with suspected primary infections was evaluated by comparing the results with the toxoplasma serology profile performed at the Research Institute, PAMF. A total of 50 samples from pregnant women were fully characterized; half were collected at >16 weeks of gestation and the other half at 7<16 weeks of gestation. All 50 samples were positive for toxoplasma IgM by the PAMF ELISA. Forty-four of the 50 positive samples (88%) had either an acute or an equivocal AC/HS pattern, and 6 had a nonacute pattern (Table 3). Only 28 of 50 (56%) of the positive samples had high-IgG-avidity antibodies, which essentially rule out recent infection. These included all 6 samples with the nonacute AC/HS pattern and 18 of 20 of the samples with an equivocal AC/HS pattern. Thus, these two confirmatory methods were generally in agreement; only four samples with high avidity had an acute AC/HS pattern. Using the BioPlex 2200, 34 of 50 of the PAMF samples were positive. For the 16 BioPlex 2200 IgM-negative samples, 15 (94%) exhibited high IgG avidity results (Fig. 1). Furthermore, 32 of 34 BioPlex toxoplasma IgM-positive samples had either an acute or an equivocal AC/HS pattern, representing 94% agreement (Table 3). In summary, specimens that were positive by the PAMF IgM test and negative by the BioPlex toxoplasmosis IgM test were in 15 of 16 cases associated with nonacute infection, representing more than half of the high-avidity samples in this study. When used as a screening test, the BioPlex method might significantly reduce the number of IgM-positive specimens that would require additional testing and ultimately be judged inconsistent with acute infection.

TABLE 3. Comparison of the AC/HS and VIDAS avidity test results for 50 samples from pregnant women

<table>
<thead>
<tr>
<th>VIDAS avidity test results</th>
<th>AC/HS pattern (n = 50)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acute (n = 24)</td>
</tr>
<tr>
<td>Low</td>
<td>16 (15)</td>
</tr>
<tr>
<td>Equivocal</td>
<td>4 (4)</td>
</tr>
<tr>
<td>High</td>
<td>4 (2)</td>
</tr>
</tbody>
</table>

*The numbers in parentheses refer to BioPlex 2200 toxoplasma-positive samples among the corresponding groups. The AC/HS test uses methanol or acetone-fixed and formalin-fixed tachyzoite preparations that express antigenic determinants found early following acute infection (AC antigen) or in the later stages of infection (HS antigen), respectively.

There is an urgent need for a test that can determine the true nature of toxoplasma infection given the long half-life of some IgM antibodies (1, 4, 12). Although several test kits are commercially available in the United States and Europe for the diagnosis of T. gondii infections, their results are less than optimal, leading to misdiagnosis and unnecessary treatment of the patient and/or termination of the pregnancy (24). An overall specificity of 99.3% was observed among healthy donor and pregnant donor samples in the present study. In the United States, the incidence of acute toxoplasma infection during pregnancy has been estimated to be approximately 0.2 to 1.0%, although in ~90% of cases it goes unrecognized. An evaluation of six commercial kits for the detection of human IgM antibodies to toxoplasma using toxoplasma serology laboratory results as the “gold standard” scored the Centers for Disease Control and Prevention IgM assay at 99.1% specificity, with other kit performances between 77.5 and 98.6% (24). Newer serological tests that help to discriminate between recent and distant infection, including the differential hemagglutination AC/HS test and avidity of toxoplasma IgG antibodies, have been introduced. While these tests are not FDA cleared for commercial use in the United States, such tests are recommended for all IgM-seropositive patients and are performed by the Research Institute, PAMF, a reference laboratory. However, these tests show a high number of equivocal results, which often lead to repeat testing (12). Approximately 32% (11 of 34) of the BioPlex IgM-positive samples tested equivocal in the AC/HS test, and 12% (4 of 34) were equivocal in the IgG avidity test. Comparison of IgG avidity test results between the PAMF ELISA and the BioPlex 2200 toxoplasma IgM-positive samples reflects a higher percentage of high-avidity samples (56%) among PAMF ELISA toxoplasma-positive samples than among BioPlex toxoplasma IgM-positive samples (38%; 13 of 34). However, such an anomaly is mainly due to the fact that 15 of 16 toxoplasma IgM-negative samples had high-IgG-avidity antibodies and hence were excluded from the initial calculation. Collectively, these results are in agreement with the published study showing higher prevalence of high-IgG-avidity antibodies among ELISA positive samples obtained during the first trimester (12). Additional evidence showing the efficacy of the BioPlex assay was demonstrated by the fact that over 70% of toxoplasma IgM-positive samples exhibited an acute AC/HS pattern after the high-avidity samples were excluded. A similar observation was made with the PAMF ELISA positive samples as well.

One explanation for the selective characteristics of the Bio-
Plex 2200 assay is a difference in performance due to the unique characteristics of a particular antigen (15). A study of several toxoplasma recombinant antigens using IgG ELISA demonstrated small differences in reactivity towards sera representing recently acquired and chronic infections (18). However, no recombinants were used in our study. Further, preliminary experiments with natural antigens from multiple sources, including harvested tachyzoites prepared in-house, did not show any change in this selective behavior. Choice of the appropriate antigen, purification of the antigen, and loading of the antigen on the solid phase all influenced specificity towards nonreactive samples but did not influence the selective lack of response to samples that were positive by ELISA methods. Therefore, the selective behavior may be characteristic of the assay format rather than a characteristic of the antigen. Under the conditions employed, the quantity of antigen used in the BioPlex 2200 assay is <10% of the quantity used for ELISA plates, and the incubation time is short. It is possible, therefore, that the selective response reflects differences in the affinity of the different antibodies under the test condition.

The Bio-Rad BioPlex 2200 toxoplasma IgM assay offers several advantages over other ELISA methods because it is a rapid, accurate, high-throughput assay with the potential for multiplexed analyses. The use of multiplexed immunoassays, based on flow cytometry, has become routine for the measurement of cytokine levels (9). In a review published in The Lancet in 2002, Jani et al. proposed that such technology could be useful for the diagnosis and surveillance of infectious diseases, even in a resource-poor setting (7). Similarly, the IgM method for detecting toxoplasma described here could be combined with additional IgM methods for detecting rubella, cytomegalovirus, and herpes to facilitate maternal screening; a matching profile of IgG antibody is also possible. The reduced sensitivity to IgM from samples with high-avidity IgG may lead to a substantial reduction in the number of IgM-positive samples that require follow-up testing. This study represents the first detailed comparison of a flow microsphere immunoassay for IgM where the sensitivity and specificity were evaluated against established reference methods, as well as the first evaluation of performance with specimens representing both acute and non-acute infection.

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REFERENCES