Multiplex Detection of IgM and IgG Class Antibodies to *Toxoplasma gondii*, Rubella Virus, and Cytomegalovirus Using a Novel Multiplex Flow Immunoassay

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The goal of this study was to evaluate the BioPlex 2200 *Toxoplasma*, rubella, and cytomegalovirus (CMV) (ToRC) IgG and IgM multiplex immunoassays (Bio-Rad Laboratories, Hercules, CA) and compare the results to those of conventional testing by enzyme immunoassay (EIA) and enzyme-linked fluorescent assay (ELFA). Serum specimens (*n* = 600) submitted for routine ToRC IgG and IgM testing by EIA (SeraQuest, Doral, FL; Diamedx, Miami, FL) or ELFA (Vidas; bioMérieux, Durham, NC) were also tested by the BioPlex ToRC multiplex immunoassays. Samples showing discordant results were retested by both methods, with further discrepancies being arbitrated by a third assay. Following repeat testing, the BioPlex *Toxoplasma*, rubella, and CMV IgG assays demonstrated agreements of 98.7 (592/600 specimens), 93.3 (560/600 specimens), and 98.3% (590/600 specimens), respectively, while the ToRC IgM assays yielded agreements of 91.2 (547/600 specimens), 87.3 (524/600 specimens), and 95.2% (571/600 specimens), respectively. The BioPlex ToRC IgG assays provided results comparable to EIA/ELFA results, with kappa coefficients showing near-perfect agreement for the *Toxoplasma* (*κ* = 0.94) and CMV (*κ* = 0.97) IgG assays and substantial agreement for the rubella IgG assay (*κ* = 0.66). The BioPlex ToRC IgM assays showed lower specificity with only slight agreement for *Toxoplasma* IgM (*κ* = 0.07), poor agreement for rubella IgM (*κ* = −0.03), and moderate agreement for CMV IgM (*κ* = 0.55). Both the BioPlex IgG and IgM assays reduced turnaround time (1.7 h versus 5.5 h by EIA/ELFA for 100 specimens) and eliminated the necessity to manually pipette or aliquot specimens prior to testing.

Congenital infections caused by *Toxoplasma gondii*, rubella, and cytomegalovirus (CMV) are a significant cause of neonatal mortality and childhood morbidity worldwide (6, 18, 21). Due to their nonspecific clinical manifestations and the importance of early recognition of *in utero* infection, serologic screening for these pathogens has been considered a routine practice in many parts of the world (11). Conventional methods for the detection of antibodies to *Toxoplasma*, rubella, and CMV (ToRC) include immunofluorescence (IFA), enzyme immunoassay (EIA), and enzyme-linked fluorescent assay (ELFA). These techniques have been used for years in both diagnostic and screening protocols for ToRC infection and have demonstrated reliable performance (5, 10, 14, 22). However, these methods have certain limitations, including low throughput, significant hands-on time, and in the case of IFA, a subjective interpretation of results.

Recently, multiplex flow immunoassay (MFI) technology emerged as a novel approach to assess the serologic response to various infectious diseases (3, 4, 13). This technology is similar to traditional EIA but allows for the simultaneous detection and identification of multiple analytes in a single reaction. MFI technology uses a liquid suspension array of up to 100 unique microspheres (5- to 6-μm beads), each conjugated with a different capture molecule (e.g., antibody, antigen, nucleic acid). Each capture analyte is detected and quantitated following the addition of a fluorescently labeled reporter molecule (e.g., phycoerythrin) whose emission is measured by a flow-based detector. In 2009, Bio-Rad Laboratories (Hercules, CA) received FDA clearance for a ToRC IgG immunoassay based on MFI technology. In addition, Bio-Rad has developed a prototype ToRC IgM assay for use in cases of suspected acute infection. These assays are fully automated on the BioPlex 2200 automated analyzer (Bio-Rad Laboratories), allowing for a high-throughput analysis of the ToRC IgG and IgM class antibody response.

Due to increasing test volumes (~20% in the past 5 years) and the limitations of conventional methods (e.g., low throughput, increased hands-on time, and the requirement to aliquot samples prior to testing), we undertook a study to evaluate the BioPlex ToRC IgG and IgM immunoassays using clinical serum samples. The goal of this study was to compare the results of the BioPlex to routine testing by EIA/ELFA, using a third assay to arbitrate discordant results.

MATERIALS AND METHODS

Study design. Prospective serum specimens (*n* = 600) submitted to our reference laboratory for routine ToRC IgG and IgM testing by EIA (SeraQuest, Doral, FL; Diamedx, Miami, FL) or ELFA (Vidas; bioMérieux, Durham, NC) were also tested by the BioPlex ToRC IgM and IgG immunoassays using the BioPlex 2200 automated analyzer (Bio-Rad). Other specimen types, including cord blood samples, were not included in this evaluation. Specimens showing discordant results after initial testing were tested again by both EIA/ELFA and the BioPlex analyzer using the same freeze-thaw cycle of the sample, with further discrepancies being arbitrated by a third assay. The study protocol was reviewed and approved by the institutional review board at Mayo Clinic.

Enzyme-linked fluorescent assay. Routine IgG and IgM testing for CMV and *T. gondii* was carried out by ELFA (Vidas; bioMérieux) according to the man-
TABLE 1. Comparison of the BioPlex ToRC IgG assays to routine testing by EIA/ELFA using prospective serum specimens (n = 600)*

| BioPlex assay and result | No. of specimens tested by EIA/ELFA that were: | % sensitivity (95% CI) | % specificity (95% CI) | % agreement (95% CI) | Kappa coefficient | PPV (%) | NPV (%) |
|--------------------------|-----------------------------------------------|------------------------|------------------------|----------------------|-------------------|---------|---------|
| Toxoplasma IgG           | Positive                                      | 63 (2°) 6            | 100.0 (93.1, 100)      | 99.6 (98.5, 99.9)    | 98.7 (97.3, 99.4)  | 0.94    | 96.9    | 100     |
|                          | Negative                                      | 0 252 0             |                        |                      |                   |         |         |         |
|                          | Equivocal                                     | 0 0 1               |                        |                      |                   |         |         |         |
| Rubella IgG              | Positive                                      | 520 2° 1            | 95.2 (93.1, 96.8)      | 73.5 (59.6, 83.9)    | 93.3 (91.0, 95.1)  | 0.66    | 99.6    | 78.3    |
|                          | Negative                                      | 10° 36 1            |                        |                      |                   |         |         |         |
|                          | Equivocal                                     | 16 11 4             |                        |                      |                   |         |         |         |
| Cytomegalovirus IgG      | Positive                                      | 336 2° 0            | 99.1 (97.3, 99.8)      | 99.2 (97.0, 100)     | 98.3 (96.9, 99.1)  | 0.97    | 99.4    | 98.8    |
|                          | Negative                                      | 3° 254 3            |                        |                      |                   |         |         |         |
|                          | Equivocal                                     | 0 0 0               |                        |                      |                   |         |         |         |

* CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value.

b For the BioPlex Toxoplasma IgG assay, results are compared to those from the Toxoplasma IgG enzyme-linked fluorescent assay (ELFA). For the BioPlex rubella IgM assay, results are compared to those from the rubella IgM enzyme immunoassay (EIA) (SeraQuest). For the BioPlex cytomegalovirus IgG assay, results are compared to those from the cytomegalovirus IgG EIA.

c Both of these serum samples were negative by the Sabin-Feldman dye test at the Palo Alto Medical Foundation Toxoplasma laboratory.

d One of these two serum samples was equivocal and one of these two samples was negative by Diamedix rubella IgG EIA.

e Three of these 10 serum samples were equivocal and seven of these 10 samples were negative by Diamedix rubella IgG EIA.

f All three of these serum samples were negative by Diamedix CMV IgG EIA.

g Agreement between EIA/ELFA and the BioPlex ToRC IgG assays. To measure agreement, the results obtained by EIA/ELFA and the BioPlex assays were compared following testing of 600 serum specimens. The BioPlex Toxoplasma, rubella, and CMV IgG assays demonstrated an agreement of 98.7% (592/600 specimens), 93.3% (560/600 specimens), and 98.3% (590/600 specimens), respectively, with the results obtained by EIA/ELFA (Table 1). Kappa coefficients showed near-perfect agreement for the Toxoplasma (κ = 0.94) and CMV (κ = 0.97) IgG assays and substantial agreement for the rubella IgG assay (κ = 0.67) (Table 1). Specimens showing discordant IgG results between EIA/ELFA and the BioPlex assays were analyzed by a third assay. Among two serum samples that were BioPlex positive and ELFA negative for Toxoplasma IgG, both were shown to be negative for IgG by the Sabin-Feldman dye test at PAMF. For specimens with discordant rubella or CMV IgG results, the arbitrating method (ELFA) resolved 7/12 (58.3%) and 3/5 (60.0%) results, respectively, in favor of the BioPlex (Table 1). Following resolution of discordant results, the BioPlex ToRC IgG assays showed an overall adjusted agreement of 98.7% for Toxoplasma IgG, 94.5% for rubella IgG, and 98.8% for CMV IgG.
TABLE 2. Comparison of the BioPlex ToRC IgM assays to routine testing by EIA/ELFA using prospective serum samples (n = 600) a

| BioPlex assay and result | No. of specimens tested by EIA/ELFA b that were: | % sensitivity (95% CI) | % specificity (95% CI) | % agreement (95% CI) | Kappa (95% CI) | PPV (%) | NPV (%) |
|-------------------------|-------------------------------------------------|------------------------|------------------------|----------------------|--------------|---------|---------|
| Toxoplasma IgM           | Positive                                         | 100.0 (29.0, 100)      | 91.1 (86.9, 93.2)      | 91.2 (88.6, 93.2)    | 0.07 (0.0, 1) | 4.9     | 100     |
|                         | Negative                                         | 0.0 (0, 34.5)          | 88.8 (86, 91.1)        | 87.3 (84.4, 89.8)    | -0.03 (0.0, 0) | 98.3    |         |
|                         | Equivocal                                        | 0.03 (0.0, 0.03)       | 99.7 (99.4, 99.8)      | 99.6 (99.4, 99.8)    | 0.55 (0.4, 0.65) | 43.3    | 99.8    |
| Rubella IgM              | Positive                                         | 96.5 (94.7, 97.8)      | 95.2 (93.1, 96.3)      | 95.2 (93.1, 96.3)    | 0.55 (0.4, 0.65) | 43.3    | 99.8    |
|                         | Negative                                         | 0.0 (0, 34.5)          | 88.8 (86, 91.1)        | 87.3 (84.4, 89.8)    | -0.03 (0.0, 0) | 98.3    |         |
|                         | Equivocal                                        | 0.03 (0.0, 0.03)       | 99.7 (99.4, 99.8)      | 99.6 (99.4, 99.8)    | 0.55 (0.4, 0.65) | 43.3    | 99.8    |
| Cytomegalovirus IgM      | Positive                                         | 96.5 (94.7, 97.8)      | 95.2 (93.1, 96.3)      | 95.2 (93.1, 96.3)    | 0.55 (0.4, 0.65) | 43.3    | 99.8    |
|                         | Negative                                         | 0.0 (0, 34.5)          | 88.8 (86, 91.1)        | 87.3 (84.4, 89.8)    | -0.03 (0.0, 0) | 98.3    |         |
|                         | Equivocal                                        | 0.03 (0.0, 0.03)       | 99.7 (99.4, 99.8)      | 99.6 (99.4, 99.8)    | 0.55 (0.4, 0.65) | 43.3    | 99.8    |

a CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value.
b For the BioPlex Toxoplasma IgM assay, results are compared to those from the Toxoplasma IgM enzyme-linked fluorescent assay (ELFA). For the BioPlex rubella IgM assay, results are compared to those from the rubella IgM enzyme immunoassay (EIA) (Diamedix). For the BioPlex cytomegalovirus IgM assay, results are compared to those from the cytomegalovirus IgM ELFA.

c All 39 of these serum samples tested negative by IgM EIA at the Palo Alto Medical Foundation Toxoplasma laboratory.
d Fourteen of these 53 serum samples were equivocal, 24 were negative, and 14 were positive by SeraQuest rubella IgM EIA.
e All nine of these serum samples were negative by SeraQuest rubella IgM EIA.
f Two of these 17 serum samples were equivocal, 14 were negative, and one was positive by Diamedix CMV IgM EIA.
g This sample was negative by Diamedix CMV IgM EIA.

Agreement between EIA/ELFA and the BioPlex ToRC IgM assays. The results from the BioPlex Toxoplasma, rubella, and CMV IgM assays demonstrated an agreement of 91.2% (547/600 specimens), 87.3% (524/600 specimens), and 95.2% (571/600 specimens), respectively, with the results obtained by EIA/ELFA (Table 2). Kappa coefficients suggested slight agreement between BioPlex and EIA/ELFA for the Toxoplasma IgM assay (κ = 0.07), poor agreement for the rubella IgM assay (κ = −0.03), and moderate agreement for the CMV IgM assay (κ = 0.55) (Table 2). Specimens showing discordant results after repeat testing by EIA/ELFA and the BioPlex assays were analyzed by a third assay. Among 39 specimens that were BioPlex positive and ELFA negative for Toxoplasma IgM, all 39 were shown to be negative for IgM by EIA analysis at PAMF. For specimens with discordant rubella or CMV IgM results, the arbitrating method (EIA) resolved 23/62 (37.1%) and 2/18 (11.1%) results, respectively, in favor of the BioPlex assay (Table 2). Following resolution of discordant results, the BioPlex ToRC IgM assays showed an overall adjusted agreement of 91.2% for Toxoplasma and rubella IgM and 95.5% for CMV IgM.

DISCUSSION

Prenatal screening for antibodies to T. gondii, rubella, CMV, herpes simplex virus type 1 (HSV-1) and HSV-2, and other agents (e.g., syphilis) is a routine practice in many parts of the world and is commonly referred to by the acronym TORCH. This screening protocol is most often used to identify pregnant mothers at risk of transmitting viral or protozoan infections in utero to the fetus or to evaluate newborns presenting with nonspecific, unexplained symptoms thought to be due to infection. Although TORCH infections are a significant cause of morbidity and mortality worldwide (6), the implementation of widespread TORCH screening programs has been questioned due to several factors, including (i) potential overuse, (ii) lack of consistent and reliable serologic methods, (iii) cost, and (iv) misinterpretation of results (1, 8, 12, 16). For example, the presence of TORCH IgG class antibodies in the mother does not differentiate between past exposure (i.e., low risk of congenital infection) and recent, acute infection (i.e., increased risk of congenital infection). Furthermore, the detection of TORCH IgM class antibodies is often interpreted as a recent infection, even though IgM antibodies may persist for months to years following exposure (17). It is important to underscore that prenatal screening for IgM class antibodies (e.g., to the TORCH complex or other infectious agents) should be limited to select situations in which the incidence of disease and pretest probability justify testing. The routine practice of screening for IgM class antibodies during pregnancy may lead to numerous false-positive results, which can cause needless worry as well as unnecessary follow-up testing and treatment (2).

Despite these considerations, serologic testing for members of the TORCH complex has been shown to be a valuable diagnostic tool when ordered judiciously (7, 19) and continues to be a common test request for clinical laboratories. For example, in 2005 our reference laboratory received 78,625 serum samples for Toxoplasma, rubella, and CMV serologic testing, and in 2009 that number increased to 98,538, representing an ~20% increase in test volumes. These data suggest that in the coming years, clinical laboratories will require accurate, rapid, and high-throughput assays to meet the expanding demand for ToRC serologic testing.

In this study, we evaluated the BioPlex ToRC IgG (FDA-approved) and IgM (prototype) multiplex immunoassays and compared the results to those obtained by routine testing by EIA and ELFA. Other routine components of the TORCH...
serology profile (e.g., anti-HSV-1 and -2 IgG and syphilis IgG) are offered as separate tests on the BioPlex platform, and evaluations of these assays have been previously published (4, 9). In this report, our findings showed that the BioPlex Toxoplasma, rubella, and CMV IgG assays demonstrated 98.7, 93.3, and 98.3% agreement, respectively, with routine testing by EIA/ELFA (Table 1). Kappa coefficients suggested near-perfect agreement between EIA/ELFA and the BioPlex T. gondii (κ = 0.94) and CMV (κ = 0.97) IgG assays and substantial agreement for the rubella IgG test (κ = 0.66) (Table 1). In contrast, our statistical analyses suggested only slight agreement between EIA/ELFA and the BioPlex T. gondii IgM assay (κ = 0.07), poor agreement for rubella IgM (κ = −0.03), and moderate agreement for CMV IgM (κ = 0.55) despite overall percent agreements of 91.2, 87.3, and 95.2%, respectively (Table 2). Interestingly, we reviewed the numerical (i.e., raw) data for the BioPlex ToRC IgM results and identified that an adjustment of the positive cutoff from ≥1.1 to ≥1.8 would improve the specificity of each BioPlex IgM test without negatively impacting sensitivity (data not shown). For example, an increase in the cutoff to ≥1.8 would reduce the number of BioPlex Toxoplasma IgM false-positive results from 39/600 (6.5%) to 4/600 (0.67%). Similarly, this modification would reduce the number of rubella IgM false-positive results from 53/600 (8.8%) to 17/600 (2.8%), and for CMV IgM the number would drop from 17/600 (2.8%) to 5/600 (0.83%). Despite these improvements in specificity, falsely positive IgM results are inevitable by any serologic method when testing is performed on asymptomatic patients, particularly in the healthy pregnant population (2). Therefore, it is our opinion that the use of IgM in evaluating patients for potential TORCH-related infection be limited to those cases in which the prevalence of disease and the pretest probability justify testing.

This study has several limitations. First, the conclusions that can be made regarding the clinical sensitivity and specificity of the BioPlex ToRC assays are limited by the lack of available clinical information (e.g., clinical manifestations, treatment decisions). Samples tested in this study were submitted to our reference laboratory without corresponding clinical data, so a correlation of the test results to disease status was not possible. Second, the number of IgM-positive samples in this study was very low (especially for Toxoplasma and rubella), and this limits the conclusions that can be made regarding the sensitivity of these tests. Third, we did not compare multiple lots of test reagents or evaluate multiple BioPlex 2200 instruments, so the potential for inter- and intralaboratory variability was not assessed.

In conclusion, we have demonstrated that the BioPlex ToRC IgG immunoassays show comparable performance to routine testing by EIA/ELFA. However, the BioPlex ToRC IgM tests showed lower overall specificity, and modifications to these assays, such as an adjustment of the analytic cutoff, may be required to improve specificity. This is especially important given the significant clinical implications of a positive ToRC IgM test. Despite these important considerations, the BioPlex ToRC immunoassays offer several advantages over conventional methods in terms of laboratory workflow and testing throughput. First, the BioPlex ToRC assays have the capacity to test for up to six analytes (3 IgM, 3 IgG), using only two samplings per serum specimen. This may reduce both sample volume requirements and aliquot errors. Second, the BioPlex incorporates three internal controls into each reaction, allowing for the verification of specimen addition, detector performance, and lack of nonspecific binding. Finally, the BioPlex allows for a more rapid (1.7 h versus 5.5 h [EIA/ELFA] for 100 samples) and high-throughput (~350 samples versus 160 samples [EIA/ELFA] per 9 h) analysis of the ToRC IgM/IgG serologic response. This may prove beneficial for high-volume clinical laboratories experiencing significant increases in the number of specimens submitted for ToRC serologic testing.

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