Development and Clinical Evaluation of a Recombinant-Antigen-Based Cytomegalovirus Immunoglobulin M Automated Immunoassay Using the Abbott AxSYM Analyzer

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A new microparticle enzyme immunoassay (MEIA), the Cytomegalovirus (CMV) Immunoglobulin M (IgM) test, was developed on the Abbott AxSYM analyzer. This test uses recombinant CMV antigens derived from portions of four structural and nonstructural proteins of CMV: pUL32 (pp150), pUL44 (pp52), pUL83 (pp65), and pUL80a (pp38). A total of 1,608 specimens from random volunteer blood donors (n = 300), pregnant women (n = 1,118), transplant recipients (n = 6), and patients with various clinical conditions and disease states (n = 184) were tested during development and evaluation of this new assay. In a preliminary clinical evaluation we tested specimens collected prospectively from pregnant women (n = 799) and selected CMV IgM-positive archived specimens from pregnant women (n = 39). The results from the new CMV IgM immunoassay were compared to the results of a consensus interpretation of the results obtained with three commercial CMV IgM immunoassays. The results for specimens with discordant results were resolved by a CMV IgM immunoblot assay. The relative sensitivity, specificity, and agreement for the AxSYM CMV IgM assay were 94.29, 96.28, and 96.19%, respectively, and the resolved sensitivity, specificity, and agreement were 95.83, 97.47, and 97.37%, respectively. We also tested serial specimens from women who experienced seroconversion or a recent CMV infection during gestation (n = 17) and potentially cross-reactive specimens negative for CMV IgM antibody by the consensus tests (n = 184). The AxSYM CMV IgM assay was very sensitive for the detection of CMV IgM during primary CMV infection, as shown by the detection of CMV IgM at the same time as or just prior to the detection of CMV IgG. Specimens from individuals with lupus (n = 16) or parovirus B19 infection (n = 6) or specimens containing hyper IgM (n = 9), hyper IgG (n = 8), or rheumatoid factor (n = 55) did not cross-react with the AxSYM assay. One specimen each from individuals infected with Epstein-Barr virus (n = 26), measles virus (n = 10), herpes simplex virus (n = 12), or varicella-zoster virus (n = 13) infection, one specimen from an influenza vaccinee (n = 14), and one specimen containing antinuclear antibody cross-reacted with the assay. The overall rate of cross-reactivity of the specimens with the assay was 3.3% (6 of 184). The AxSYM CMV IgM assay is a sensitive and specific assay for the detection of CMV-specific IgM.

Human cytomegalovirus (CMV) is a herpesvirus which is ubiquitously distributed in the human population. Although rarely pathogenic in immunocompetent individuals, the virus poses a significant health threat to immunocompromised individuals and is a significant cause of morbidity and mortality in organ allograft and bone marrow transplant recipients (7, 23, 29). Pregnant women are also a risk group for this virus as CMV is the most common cause of congenital infection. Since infections with CMV either are asymptomatic or are accompanied by symptoms not specific for CMV, laboratory diagnostic methods are used to diagnose CMV infection. Diagnosis of CMV infection can be accomplished by detection of virus in several body fluids such as blood, urine, or saliva or indirectly through serology. Serological tests are used to diagnose primary CMV infection by the detection of antibodies in a previously seronegative individual. In the absence of seroconversion, CMV-specific immunoglobulin M (IgM) is a sensitive and specific indicator of active or recent CMV infection, while it is very often produced during viral reactivation in immunocompromised individuals (1, 19).

Detection of CMV-specific IgM is most commonly done by using preparations of the virus or viral lysate in an enzyme-linked immunosorbent assay (ELISA) (11, 30). Poor agreement among these tests has been found (13, 14), presumably due to the different viral preparations used in the various commercial kits. The key serological targets for detection of CMV-specific IgM comprised both the structural pUL32 (pp150), pUL83 (pp65), and pUL80a (pp38) (8, 9, 10) viral proteins and the nonstructural pUL57 (p130) and pUL44 (pp52) (24, 31) viral proteins. Variations in the relative amounts of these antigens produced during growth and purification of the virus can result in different relative compositions of the structural and nonstructural viral antigens used in the
various IgM tests. The use of nonstandardized viral antigens to capture CMV IgM can contribute to interassay variation. In contrast, purified recombinant proteins and peptides can be consistently manufactured and optimized to capture CMV-specific IgM, which can improve CMV assay standardization (5, 12, 32). In this work we describe the development and preliminary clinical evaluation of the first fully automated, commercially available, recombinant antigen-based CMV IgM immunoassay.

(A portion of this work was presented at the Abbott-sponsored symposium entitled New Developments in the Diagnosis of CMV, Toxo, and Rubella Infection, held in Venice, Italy, May 1998.)

MATERIALS AND METHODS

Cloning and expression of CMV genes. All CMV gene fragments that encode antigen were obtained by PCR amplification with PCR primers designed to

i) Specimens from pregnant women. Fresh maternal serum specimens were collected prospectively from pregnant women from Swiss (n = 599; Dianalab S.A., Geneva, Switzerland) and U.S. (n = 200; Eastern Virginia Medical School, Norfolk, Va.) populations. The average age of the women in the Swiss population was 31.1 years, with 53.1, 29.9, and 17.1% of the specimens drawn during the first, second, and third trimesters, respectively. The average age of the U.S. population was 25.9 years, with 40.0 and 60.0% of the specimens drawn during the first and second trimesters, respectively. These specimens were tested with the AxSYM instrument prior to freezing and were used to evaluate assay sensitivity.

ii) Selected positive specimens. Selected frozen serum specimens (n = 39) from a Swiss population of pregnant women positive for CMV IgM antibody as determined by the IMx CMV IgM assay (Abbott Laboratories) were used to evaluate assay specificity. Three of these specimens overlap with the specimens listed in i).

iii) Selected serial specimens. A total of 17 serial specimens from three suspected CMV-IgM-positive women were tested to evaluate the kinetics of appearance and disappearance of CMV-specific IgM. These women were pregnant during the evaluation. These specimens were also tested by the IMx CMV IgM and AxSYM CMV IgG assays.

iv) Potentially cross-reactive specimens. Potentially cross-reactive specimens, i.e., specimens known to be seropositive for a variety of specific infections and/or medical conditions, were tested to determine potential cross-reactivity in the assay. The potentially cross-reactive specimens were positive for antinuclear antibodies (n = 5; Gamma Diagnostics, Division of Compuware, Farmington Hills, Mich.; Immunochrom, Inc.), Epstein-Barr virus (n = 5; General Diagnostics, Fairfield, N.J.; Sanofi, France), human immunodeficiency virus (n = 6; Hoffman-La Roche, U.S.); hepatitis A virus (n = 10; measles virus (n = 26; Abbott Laboratories, Abbott Park, Ill.) are automated immunoassay analyzers that use microparticle technology.

The AxSYM and IMx instruments (Abbott Laboratories, Abbott Park, Ill.; BioClinical Partners, Inc.), and Hyper IgG (n = 8; BioClinical Partners, Inc.) were from influenza vaccines (n = 14; Cash Blood Bank, Piscataway, N.J.). These specimens were characterized for neutralization reactivity using the appropriate methodologies to verify the clinical condition or disease state. Neutralization reactivity (Abbott Manufacturing Inc., Abbott Park, Ill.,) was used to neutralize RF antibodies.

v) Precision panels. Serum and plasma panels were prepared to evaluate the precision of the AxSYM assay. Four panel members were negative for CMV IgM, four panel members were low positive (index values, <1.000) for CMV IgM, and four panel members were positive for CMV IgM. The low-positive and positive serum and plasma panel members were prepared artificially by spiking CMV-negative serum or plasma with CMV IgM-positive serum. The precision of the AxSYM assay. The method of Wunderli et al. (34) was used for the detection of the immediate-early antigen in human embryonic fibroblasts.

Commercial CMV IgM assays and consensus interpretation. The assay cutoff and the relative performance characteristics of the AxSYM assay were determined by testing all specimens with three commercial tests (consensus result) for the presence of CMV IgM: the Ampligen assay (QCP, Inc.), the AxSYM assay, and the IMx assay. The Ampligen assay was chosen for this performance evaluation as it had been shown to agree reasonably well with the CMV IgM immunoblot assay result (16, 17) (data not shown). Specimens that were positive or negative by the AxSYM assay and discordant by the consensus interpretation were further resolved by CMV IgM immunoblot testing (17).

Statistical methods. Sensitivity and specificity were calculated as described by Griner et al. (6). Agreement was calculated as follows: (TP + TN)/(TP + TN + FP + FN) × 100, where TP is the number of true-positive specimens, TN is the number of true-negative specimens, FP is the number of false-positive specimens, and FN is the number of false-negative specimens. The 95% confidence interval (CI) determined for relative sensitivity, specificity, and agreement was based on the binomial distribution by using the STATXACT-3 software (J. A. Statistical analysis was used to assist the determination of the preliminary cutoff for the AxSYM assay (25, 35). The precision of the AxSYM assay was determined by use of National Committee for Clinical Laboratory Standards protocol EP-T2 as a guideline (22). The standard deviation (SD) and percent coefficient of variance (CV) were determined by a variance component analysis for a random-effects model (2, 27). Negative variance components were set equal to zero.
RESULTS

Determination of assay cutoff and preliminary performance evaluation. An assay cutoff was established by testing 572 specimens from the following categories: 199 specimens from pregnant women, 300 specimens from random volunteer whole-blood donors, and 73 suspected positive specimens from heart transplant recipients and pregnant women. These specimens were tested by the AxSYM CMV IgM assay and by three other commercial assays (Gull CMV IgM ELISA, Trinity Biotech/Centocor CAPTIA CMV-M, and Abbott CMV-M EIA). The results from the AxSYM assay were then compared to the consensus interpretation. ROC analysis was used to assist in the determination of the preliminary cutoff. ROC analysis depicts the overlap between the negative and positive distributions by tabulating sensitivity and specificity over a range of cutoff values. Specimens with a consensus interpretation of none or equivocal were excluded from the ROC analysis. Specimens that were tested by the AxSYM assay and that had index values greater than or equal to the cutoff were classified as positive, and specimens that had index values less than the cutoff were classified as negative. The ROC classification summaries for the AxSYM assay are presented in Table 1. As shown in Table 1, the ROC profile indicates a minimum distance at a cutoff of 0.400 as the optimum point where both sensitivity and specificity were maximized. In order to further optimize the assay cutoff subsequent to the ROC analysis, the cutoff was raised to 0.500 and a normal approximation of the cutoff would improve assay specificity without negatively affecting assay sensitivity. Comparison of the sensitivity of the AxSYM assay at a 0.500 versus a 0.400 index value cutoff indicated no statistically significant difference in assay sensitivity within a 95% CI (z = 0.917; P > 0.05). However, a statistically significant difference in assay specificity was observed at this cutoff within a 95% CI (z = 2.210; P ≤ 0.05). On the basis of these analyses, the optimum cutoff for the assay was set at an index value of 0.500.

To further improve the separation between the negative and positive populations, an equivocal zone with index values from 0.400 to 0.499 was introduced. Specimen results were then interpreted as follows. Specimens with index values less than 0.400 were considered negative for CMV IgM antibody. Specimens with index values in the range of 0.400 to 0.499 were considered equivocal. Specimens interpreted as equivocal may contain very low levels of CMV IgM antibody. Specimens with index values equal to or greater than 0.500 were considered positive for CMV IgM antibody. All specimens were then tested by the three commercial assays. The relative sensitivity, specificity, and agreement for the AxSYM CMV IgM assay are shown in Table 2. There were approximately 4 SDs from the mean for the negative population to the assay cutoff of 0.500 (data not shown). Specimens that had positive and negative results by the AxSYM CMV IgM assay but that were discordant by the consensus interpretation were tested by the CMV IgM immunoblot assay. Of the 23 discordant specimens, 21 were tested by the CMV IgM immunoblot assay. In addition, one of the six specimens which was negative by AxSYM and none by the consensus interpretation was also tested by the

<table>
<thead>
<tr>
<th>Interpretation</th>
<th>Serum sample group (no. of specimens)</th>
<th>No. of specimens with the following interpretation:</th>
<th>% Sensitivity (95% CI)</th>
<th>% Specificity (95% CI)</th>
<th>% Agreement (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Consensus</td>
<td>Pos (93)</td>
<td>68 0 14 11</td>
<td>88.31 (78.97–94.51)</td>
<td>96.93 (94.90–98.31)</td>
<td>95.68 (93.60–97.25)</td>
</tr>
<tr>
<td></td>
<td>Eqv (21)</td>
<td>4 0 15 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Neg (458)</td>
<td>9 1 442 6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resolved</td>
<td>Pos (93)</td>
<td>76 0 6 11</td>
<td>97.44 (91.04–99.69)</td>
<td>98.68 (97.16–99.52)</td>
<td>98.50 (97.07–99.35)</td>
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<td>Eqv (21)</td>
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<tr>
<td></td>
<td>Neg (458)</td>
<td>2 1 450 5</td>
<td></td>
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</tbody>
</table>

*Consensus assays: Abbott CMV-M EIA, Gull CMV IgM ELISA, and Trinity Biotech/Centocor CAPTIA CMV-M. Abbreviations: Pos, positive; Eqv, equivocal; Neg, negative; None, no interpretation. Sensitivity, specificity, and agreement are relative values for the consensus interpretation and resolved values for the resolved interpretation.
immunoblot assay. The resolved sensitivity, specificity, and agreement for the AxSYM CMV IgM assay are shown in Table 2.

The AxSYM CMV IgM and IMx CMV IgM version 2.0 assays were developed in parallel, and both use recombinant CMV antigen-coated microparticles. The main difference between these assays is that the AxSYM and IMx assay reagents are run on their respective instruments. With samples not shown). Relative specificity = 96.46% (95% CI = 94.80 to 97.16%); resolved specificity = 97.45% (95% CI = 96.05 to 98.46%).

**Relative sensitivity** = 96.43% (95% CI = 81.65 to 99.91%); resolved sensitivity = 100.00% (95% CI = 88.43 to 100.00%).

### TABLE 3. Comparison of AxSYM CMV IgM assay results to consensus and resolved interpretations

<table>
<thead>
<tr>
<th>Population and test</th>
<th>Serum specimen group (no. of specimens)</th>
<th>Consensus interpretation</th>
<th>No. of specimens</th>
<th>Resolved interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnant women (n = 799)</td>
<td>AxSYM Pos (36)</td>
<td>Pos</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>AxSYM Eqv (34)</td>
<td>Eqv</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>AxSYM Neg (729)</td>
<td>Neg</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Suspected CMV IgM-positive pregnant women (n = 39)</td>
<td>AxSYM Pos (30)</td>
<td>Pos</td>
<td>27</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>AxSYM Eqv (5)</td>
<td>Eqv</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>AxSYM Neg (4)</td>
<td>Neg</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

*Relative specificity = 96.46% (95% CI = 94.80 to 97.16%); resolved specificity = 97.45% (95% CI = 96.05 to 98.46%).

**Relative sensitivity** = 96.43% (95% CI = 81.65 to 99.91%); resolved sensitivity = 100.00% (95% CI = 88.43 to 100.00%).
by the AxSYM CMV IgM assay in patient 2. There were no clinical indications of congenital CMV infection in either pregnancy (as determined by ultrasound) or during the subsequent 2 years of postnatal follow-up. Patient 3 experienced a recent CMV infection as indicated by the presence of CMV IgM (as tested only by the IMx assay) antibodies at week 6 of gestation. The first specimen that was tested by the AxSYM CMV IgM assay was positive for CMV IgM at week 7 of gestation. This pregnancy was terminated at week 8 of gestation, and at autopsy the fetus was found to be congenitally infected with CMV (CMV early antigen positive). During a second pregnancy 5 months later, patient 3 remained positive for CMV IgM as measured by the IMx assay but negative for CMV IgM as measured by the AxSYM assay. The faster kinetics of disappearance of CMV IgM as measured by the AxSYM assay relative to that measured by the IMx assay has been subsequently confirmed with six of eight patients who experienced a recent CMV infection (data not shown).

**Evaluation of assay cross-reactivity.** Potentially cross-reactive specimens, i.e., specimens known to be seropositive for a variety of specific infections and/or medical conditions, were tested to determine potential cross-reactivity in the assay \( n = 184 \). All specimens tested were negative for CMV IgM antibody by all three commercial assays (consensus). Cross-reactivity was indicated if the specimen was positive by the AxSYM CMV IgM assay. For RF-positive specimens, cross-reactivity was indicated if the result for the specimen by the AxSYM CMV IgM assay changed from positive to negative following neutralization of the specimen with RF neutralization reagent. Specimens from individuals with systemic lupus erythematosus or parvovirus B19 infection or specimens containing Hyper IgM, Hyper IgG, or RF did not cross-react with the AxSYM assay. One specimen each from individuals infected with Epstein-Barr virus, measles virus, herpes simplex virus, or varicella-zoster virus infection, one specimen from an influenza vaccinee, and one specimen containing antinuclear antibodies cross-reacted with the assay. The overall rate of cross-reactivity of the specimens with the assay was 3.3% \( (6 \text{ of } 184) \).

**DISCUSSION**

One of the problems that the diagnostic laboratory has faced over the past 10 years is the lack of agreement between commercial tests for the detection of CMV-specific IgM \( (13, 14) \). This lack of agreement has its roots in the different viral preparations used to detect IgM antibodies to CMV. Since detection of the humoral IgM response is improved by including both structural and nonstructural viral proteins \( (8, 9, 10, 24, 31) \), the performance of the viral antigen-based tests is directly dependent on how the virus is grown and how the viral antigens are purified. Our group and others have shown that a balanced cocktail of highly purified recombinant antigens \( (12, 18) \) or peptides \( (5) \), which contain both structural and nonstructural viral antigens, can replace the virus for detection of CMV-specific IgM. In this report we describe the development of the first automated, commercially available, recombinant antigen-based CMV IgM immunoassay for the detection of CMV-specific IgM.

One of the challenges that we faced with the development of a recombinant antigen-based test is that the results of this new test would likely not agree with those of the other commercial CMV IgM tests whose results disagree with one another. A CMV IgM serological reference standard was needed to define the “truth” for a specimen with respect to the presence of CMV-specific IgM apart from virus detection. During development of the recombinant antigen-based test, we also developed two versions of a CMV IgM immunoblot assay which can be considered a reference test for CMV IgM serology \( (13, 15, 16, 17) \). The CMV IgM immunoblot assay \( (16, 17) \) was used as a benchmark for development of this recombinant antigen-based CMV IgM test on the AxSYM and IMx immunoassay analyzers. This blot was also used to select the three commercial assays \( (\text{Abbott CMV-M EIA, Gull CMV IgM ELISA, CAPTIA CMV-M}) \) which, as a consensus, were used to determine the assay cutoff and performance characteristics. The optimal cutoff established for the assay \( (\text{Tables 1 and 2}) \) was further examined during the clinical evaluation of the assay with samples from a population of pregnant women \( (\text{Table 3}) \). The resolved sensitivity and specificity for the assay presented in Table 3 are similar to those presented in Table 2, thus validating the cutoff for the assay. Statistical analysis of the precision of the assay near the cutoff indicates that the precision of the assay is sufficient to withstand false-positive or false-negative results on the basis of mere measurement variability within a 95% CI \( (\text{data not shown}) \).

The sensitivity and specificity of the AxSYM assay were examined further by testing characterized specimens. The sensitivity of the assay was examined by testing serial specimens from pregnant women who experienced seroconversion or a recent CMV infection during gestation. Our results indicate that the AxSYM CMV IgM assay can detect early seroconversion at a rate comparable to that for the viral lysate-based commercial CMV IgM assays. The sensitivities and concomitant positive reactivity rates for various commercial assays for the detection of CMV-specific IgM have been shown to vary widely \( (13, 14) \). In this study the AxSYM assay had a higher positive reactivity rate than the three commercial tests. Several studies have shown that anywhere from 5 to 15% of CMV-seropositive women excrete the virus during gestation, with higher rates of viral excretion observed in women of advanced gestational age \( (20, 29) \). The positive reactivity rate of the AxSYM assay is consistent with this percentage of women undergoing active CMV infection, as indicated by excretion of the virus. The specificity of the AxSYM assay was further evaluated by testing potentially cross-reactive specimens. Low levels of cross-reactivity were observed for the assay. Treatment of CMV IgM-positive or equivocal specimens with RF neutralization reagent was found to be unnecessary. In conclusion, the new recombinant antigen-based AxSYM CMV IgM assay is a sensitive and specific test for the detection of CMV-specific IgM.

**ACKNOWLEDGMENTS**

We thank Abbott Laboratories for funding the development and clinical evaluation of the AxSYM and IMx version 2.0 CMV IgM assays. We also thank Rita Holzman, Rong Wang, and David Ownby for the development and scale-up of the CMV recombinant antigen protein purification process and W. Wunderli for CMV antigen testing.

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