The accurate discrimination of primary cytomegalovirus (CMV) infection from reactivation or reinfection plays an important role in the clinical management of pregnant women (1–5). Primary CMV infection during pregnancy may lead to intrauterine infection of the fetus, which is associated with hearing loss, blindness, and mental retardation (6–8). In contrast, CMV reactivation or reinfection during pregnancy is rarely associated with intrauterine fetal infection (2, 7). The accurate identification of primary CMV infection is also important in managing solid organ transplant recipients, who exhibit an increased risk of organ rejection following primary CMV infection (9–11).

Over the last decade, the assessment of CMV IgG avidity has become the preferred laboratory tool for identifying patients with primary CMV infection (4, 5). IgG avidity gradually matures over time, so that IgG detected by 5 to 6 months after a primary infection exhibits high avidity; thus, a low CMV IgG avidity result is a strong indicator of primary infection within the preceding 5 to 6 months (2, 3, 6, 12, 13). CMV IgG avidity is more accurate than CMV IgM detection for identifying primary infection, since CMV IgM persists for >6 months in some patients (2, 7, 14).

In 2002, we validated a laboratory-developed CMV IgG avidity enzyme immunoassay (EIA). The experiments demonstrated low avidity in 99% of the serum samples from pregnant women collected within 120 days of the last IgG-negative serum sample (consistent with primary infection) but high avidity in 96% of the serum samples exhibiting a reactivity profile consistent with past infection (IgG positive/IgM negative) (15). Recently, however, we were forced to discontinue this assay because the CMV antigen preparation used to coat the microtiter wells is no longer available. Although several CMV IgG avidity assays have become commercially available in the United States over the last decade (16–19), most are automated instrument/reagent systems requiring a sizeable capital investment. We thus sought to determine if a commercially available CMV IgG EIA kit could be modified to accurately measure CMV IgG avidity and thus replace our laboratory-developed test.

MATERIALS AND METHODS

Serum panels. Three different serum panels were utilized for the experiments described herein. Panel 1, which was used to validate the modification of the Wampole Laboratories (Princeton, NJ) CMV IgG avidity EIA kit to measure CMV IgG avidity, consisted of 156 CMV IgG-positive/IgM-negative serum samples employed in 2002 to validate the laboratory-developed CMV IgG avidity assay. Panel 1 consisted of 470 consecutive serum samples submitted for CMV IgG avidity testing. Surprisingly, 101 serum samples were negative when screened for CMV IgG using the Wampole kit per the package insert; 98 of these 101 serum samples were tested using a CMV IgG chemiluminescent immunoassay, and only 5 were positive. Of the 369 CMV IgG-positive samples, 6% exhibited low IgG avidity, 6% exhibited intermediate avidity, and 88% exhibited high avidity; CMV IgM detection rates were inversely related to AI levels. These findings show that (i) the Wampole CMV IgG EIA can be modified to measure CMV IgG avidity, (ii) many samples are apparently submitted for avidity testing without knowledge of their CMV IgG status, and (iii) most CMV IgG-positive sera submitted for avidity testing exhibit high avidity.
CMV IgG and IgM. CMV IgG in serum panels 2 and 3 was measured using the Wampole CMV IgG EIA, per the manufacturer’s instructions; this assay is cleared by the U.S. Food and Drug Administration (FDA) for detecting CMV IgG in human serum specimens. All available panel 3 samples that were negative or equivocal for CMV IgG by the Wampole assay were tested for CMV IgG using an FDA-cleared chemiluminescent immunoassay (CIA) (Immulite, Siemens Diagnostics, Los Angeles, CA) (20). In all panel 2 samples and in CMV IgG-positive panel 3 samples, CMV IgM was measured with an FDA-cleared magnetic bead-based flow cytometric immunofluorescent assay (21), performed per the manufacturer’s instructions (BioPlex 2200; Bio-Rad Laboratories, Hercules, CA). For this study, equivocal CMV IgM results were considered positive.

Modification of the Wampole CMV IgG EIA to measure avidity. The CMV IgG-positive sera were diluted 1:21 in kit-supplied specimen diluent and added to duplicate microtiter wells; after 25 min at room temperature (RT), the well contents were discarded. Kit wash buffer was added to one of each duplicate well, and dissociating buffer (kit wash buffer containing 6 M urea [MP Biomedicals, Solon, OH]) was added to the other well (15). The well contents were discarded after 5 min at RT, and the wash procedure was repeated (including the 5-min incubation). All wells were washed once more with kit wash buffer; the assay was then finished per the package insert, and the optical density (OD) values were determined. The avidity index (AI) was calculated using the formula AI (OD for the well washed with dissociating buffer/OD for the well washed with kit wash buffer) × 100; the result was expressed as a percentage.

Statistical analysis. The correlation between the AI values obtained for panel 1 sera using the modified Wampole EIA for CMV IgG avidity and the laboratory-developed EIA was assessed using MedCalc (Ostend, Belgium) statistical software. MedCalc was also used for chi-square analysis of the proportional differences in CMV IgM detection in relation to the qualitative Wampole CMV IgG avidity assay result.

RESULTS
Validation of the Wampole CMV IgG EIA to measure avidity. Fig. 1 presents the AI values obtained in 2013 using the Wampole avidity assay plotted as a function of the AI values obtained in 2002 using the laboratory-developed avidity assay (15) for 156 cryopreserved serum samples from the original 2002 validation (panel 1). Excellent correlation was observed (correlation coefficient \( r = 0.9648, P < 0.0001 \)). Further, the high slope value (0.9778) indicated that the AI value obtained for a given serum using the Wampole avidity assay was very similar to the value obtained using the laboratory-developed avidity assay. We thus assessed the qualitative agreement between Wampole avidity assay results and laboratory-developed assay results when applying the interpretive criteria for the laboratory-developed assay to the Wampole avidity assay. These interpretive criteria are as follows: low avidity, AI of \( \leq 0.50 \); intermediate avidity, AI of 0.51 to 0.59; high avidity, AI of \( \geq 0.60 \). Table 1 presents the results of this assessment. All 78 samples with low CMV IgG avidity in the laboratory-developed assay also exhibited low avidity in the Wampole assay; these 78 serum samples were from pregnant women with documented CMV seroconversion and included 72 samples collected within 120 days of the last CMV IgG-negative sample. Of 7 serum samples with intermediate avidity in the laboratory-developed assay, 3 exhibited low avidity, and 3 exhibited high avidity. All 71 samples with high avidity in the laboratory-developed assay, 66 also exhibited high avidity in the Wampole assay, and 5 exhibited intermediate avidity; interestingly, 3 of the 5 serum samples with intermediate avidity in the Wampole assay were from pregnant women with documented CMV seroconversion and included 72 samples collected within 120 days of the last CMV IgG-negative sample. Of 7 serum samples with intermediate avidity in the laboratory-developed assay, 3 exhibited low avidity, and 3 exhibited high avidity. All 71 samples with high avidity in the laboratory-developed assay, 66 also exhibited high avidity in the Wampole assay, and 5 exhibited intermediate avidity; interestingly, 3 of the 5 serum samples with intermediate avidity.
The interassay CVs were 11% and 3%, respectively. The gender and assay was assessed using a panel of 470 consecutive serum samples to perform the newly validated Wampole CMV IgG avidity assay results for 156 serum samples used in 2002 to validate the laboratory-developed CMV IgG avidity EIA (panel 1)\(^a\)

| Laboratory-developed avidity level | Wampole assay results by avidity level | Total Wampole assay results |
|-----------------------------------|---------------------------------------|-----------------------------|
| Low                               | Low                                   | 78                          |
| Intermediate                      | Intermediate                           | 3                           |
| High                              | High                                   | 7                           |
| Total                             | Total                                  | 84                          |

\(^a\) Concordance was calculated using the above data as follows: \((78 + 3 + 66)/156 \approx 94\%\).

The precision of the Wampole avidity assay was assessed using a low-avidity serum and a high-avidity serum. Intra-assay precision was determined by testing 9 replicates of the sera within the same assay run; interassay precision was determined by testing the sera on 5 separate runs. As shown in Table 2, the intra-assay coefficient of variation (CV) values were 6% and 5%, respectively, and the interassay CVs were 11% and 3%, respectively.

Findings for sera submitted for CMV IgG avidity testing. The performance of the newly validated Wampole CMV IgG avidity assay was assessed using a panel of 470 consecutive serum samples submitted for CMV IgG avidity testing (panel 3). The gender and age distributions of the patients providing serum samples for CMV IgG avidity testing are shown in Table 3. As expected, the vast majority (427/470 [91\%]) of the specimens were supplied by women of childbearing age (15 to 49 years); 3\% of the serum samples were from females \(<15\) or \(>49\) years of age, and 6\% of the samples were from males.

Because CMV IgG positivity is a criterion for performing CMV IgG avidity testing at our facility, all sera were first screened for CMV IgG using the Wampole CMV IgG EIA kit per the manufacturer’s instructions. The CMV IgG results for the 470 serum samples are shown in Fig. 2. Surprisingly, 101 samples (21\%) were not positive for CMV IgG (96 were negative and 5 equivocal). To determine if this large percentage of samples not meeting the criterion for CMV IgG avidity testing reflected markedly reduced sensitivity of the Wampole CMV IgG EIA, all available Wampole CMV IgG-negative serum samples \((n = 98)\) were tested using a CIA for CMV IgG. Five of these 98 samples were CMV IgG positive by CIA, 3 were equivocal, and 90 were negative; 3 of the 5 samples positive by CIA were equivocal by EIA, and 4 of the 5 CIA-positive samples were submitted by a single regional reference laboratory that uses CIA to measure CMV IgG.

The Wampole CMV IgG avidity assay results for 369 consecutive Wampole CMV IgG-positive serum samples are shown in Fig. 2; 6\% of the samples exhibited low avidity, 6\% exhibited intermediate avidity, and 88\% exhibited high avidity. The CMV IgM detection rate within the three avidity groups decreased as avidity increased (Fig. 2). In pairwise comparisons, the IgM-positive percentage was significantly higher in the low-avidity group than in the high-avidity group (91\% versus 21\%; chi-square value, 52.7; \(P < 0.0001\)) and also higher in the intermediate-avidity group than in the high-avidity group (74\% versus 21\%; chi-square value, 30.2; \(P < 0.0001\)); the IgM-positive percentage did not differ significantly between the low-avidity group and the intermediate-avidity group (chi-square value, 1.3; \(P = 0.24\)).

**TABLE 2** Precision results for the Wampole CMV IgG avidity EIA\(^a\)

| Comparison type (no. of replicates) | Parameter       | Low-avidity serum | High-avidity serum |
|-----------------------------------|-----------------|-------------------|--------------------|
| Intra-assay (9)                   | Minimum         | 0.28              | 0.69               |
|                                  | Maximum         | 0.35              | 0.80               |
|                                  | Mean            | 0.32              | 0.75               |
|                                  | SD              | 0.02              | 0.04               |
|                                  | CV (%)          | 6                 | 5                  |
| Interassay (5)                   | Minimum         | 0.23              | 0.74               |
|                                  | Maximum         | 0.32              | 0.79               |
|                                  | Mean            | 0.28              | 0.76               |
|                                  | SD              | 0.03              | 0.02               |
|                                  | CV (%)          | 11                | 3                  |

\(^a\) Results represent AI values.

**TABLE 3** Patient age and gender for 470 consecutive serum samples submitted for CMV IgG avidity testing (panel 3)

| Patient age, gender | No. | Proportion (%) of total |
|---------------------|-----|-------------------------|
| Male                | 29  | 6.17                    |
| Female              | 95  | 19.83                   |
| <15                 | 4   | 0.85                    |
| 15–25               | 132 | 26.38                   |
| 26–35               | 239 | 51.49                   |
| 36–49               | 61  | 12.98                   |
| >49                 | 10  | 2.13                    |
| Total               | 470 | 100.00                  |

**DISCUSSION**

Our findings demonstrate that a commercially available CMV IgG EIA kit from Wampole Laboratories can be modified to accurately measure CMV IgG avidity. The interpretive criteria defined for our laboratory-developed CMV IgG avidity EIA were applicable to the modified Wampole assay, and thus no reporting changes were required. Further, the actual AI values obtained in the 2 assays showed excellent correlation.

The performance of the Wampole CMV IgG avidity assay was...
assessed for a panel of 470 consecutive serum samples submitted for CMV IgG avidity testing. The vast majority (91%) of these samples were from women of childbearing age. Assuming that most of these women were pregnant, this skewed gender/age distribution suggests that the test is being ordered for its intended purpose.

Our testing protocol requires that sera submitted for CMV IgG avidity testing first be tested for CMV IgG to ensure that CMV-specific IgG is indeed present in each sample (22, 23); the Wampole CMV IgG EIA kit is used per the manufacturer’s instructions for this purpose. We were surprised to find that 21% of sera submitted for CMV IgG avidity were not positive with the Wampole CMV IgG EIA. To further investigate this unexpected finding, we used a different methodology (CIA) to test for CMV IgG in all available samples exhibiting a negative or equivocal result with the Wampole CMV IgG EIA; 95% of these samples were also negative or equivocal for CMV IgG by the CIA. This finding indicates that unacceptably low EIA sensitivity does not explain the unexpectedly large number of CMV IgG-negative specimens submitted for CMV IgG avidity testing. Rather, it suggests that these sera were not tested for CMV IgG before submission, an approach that is inconsistent with recommended algorithms for diagnosing and managing primary CMV infection during pregnancy (12, 13, 22, 23). Programs are thus needed to educate physicians and laboratory personnel on the need to demonstrate CMV IgG positivity before submitting samples to an esoteric reference laboratory for CMV IgG avidity testing.

Of the 369 serum samples in panel 3 that were positive for CMV IgG by the Wampole EIA, 6% exhibited low IgG avidity, 6% exhibited intermediate avidity, and 88% exhibited high avidity. Since the vast majority of the samples were from women of childbearing age, who were assumed to be pregnant, the small proportion of samples exhibiting low or intermediate CMV IgG avidity is good news from a clinical standpoint, indicating a low frequency of primary CMV infection after conception (1–3). The frequency of low-avidity samples among CMV IgG-positive sera submitted to our esoteric reference laboratory is somewhat higher than the frequency of low-avidity samples among CMV IgG-positive sera from a population sample of women of childbearing age residing in the United States (6% versus 2%, respectively) (24). This higher rate is not unexpected, since the skewed gender/age distribution strongly suggests that a sizeable proportion of the submitted CMV IgG-positive samples were collected from pregnant patients with suspected CMV exposure.

In support of other published findings (1, 2, 24, 25), we found that the rate of CMV IgM detection among sera exhibiting low CMV IgG avidity (91%) was significantly higher than the CMV IgM detection rate among sera exhibiting high avidity (21%). Our study extends these findings by demonstrating that the CMV IgM detection rate was also significantly higher in the intermediate-avidity group than in the high-avidity group (74% versus 21%, respectively). This finding suggests that an intermediate-avidity result, like a low-avidity result, may be clinically useful for estimating the length of time since primary CMV infection.
atic prospective studies evaluating large groups of patients with known CMV IgG conversion dates are needed to accurately define the postinfection time window associated with an intermediate CMV IgG avidity result. Such patient groups include not only pregnant women but also CMV-seronegative recipients of solid organs from CMV-positive donors (2). However, immunosuppression may delay avidity maturation in transplant recipients (10), suggesting that the results for this patient group should be interpreted with caution.

While the vast majority of low-avidity serum samples were positive for CMV IgM, 9% were IgM negative. Although this finding is consistent with published data (2, 24), it raises questions about the utility of algorithms requiring CMV IgM reactivity as a criterion for CMV IgG avidity testing (12, 13, 24). This issue is addressed in a companion publication (26) in which we assess the potential impact of 4 different CMV IgM assays on a reflexive algorithm in which only IgM-positive samples are tested for IgG avidity.

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