Evaluation of Three Multiplex Flow Immunoassays Compared to an Enzyme Immunoassay for the Detection and Differentiation of IgG Class Antibodies to Herpes Simplex Virus Types 1 and 2

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The diagnosis of herpes simplex virus (HSV) infections is routinely made based on clinical findings and supported by laboratory testing using PCR or viral culture. However, in instances of subclinical or unrecognized HSV infection, serologic testing for IgG class antibodies to type-specific HSV glycoprotein G (gG) may be useful. This study evaluated and compared the performances of three multiplex flow immunoassays (AtheNA Multi-Lyte [Zeus Scientific], BioPlex 2200 [Bio-Rad Laboratories], and Plexus HerpeSelect [Focus Diagnostics]) for the simultaneous detection of gG type-specific IgG antibodies to HSV types 1 and 2 (HSV-1 and HSV-2). Serum specimens (n = 505) submitted for routine gG type-specific HSV IgG testing by enzyme immunoassay (EIA) (HerpeSelect; Focus Diagnostics) were also tested by the three multiplex flow immunoassays. Specimens showing discordant results were tested by HSV type-specific Western blotting (WB). For HSV-1 IgG, the AtheNA, BioPlex, and Plexus assays demonstrated agreements of 94.9% (479/505 specimens), 97.8% (494/505 specimens), and 97.4% (492/505 specimens), respectively, with the results of EIA. For HSV-2 IgG, the AtheNA, BioPlex, and Plexus assays showed agreements of 87.9% (444/505 specimens), 97.2% (491/505 specimens), and 96.8% (489/505 specimens), respectively, with EIA results. Timing studies showed that the AtheNA, BioPlex, and Plexus assays could provide complete analysis of 90 serum specimens in 3.1, 1.5, and 2.9 h, respectively, versus 3.1 h by EIA. These findings suggest that the gG type-specific HSV IgG multiplex immunoassays may be beneficial to high-volume clinical laboratories experiencing significant increases in the number of specimens submitted for HSV serologic testing. The evaluated systems provide comparable results to those of EIA, while reducing hands-on time and eliminating the necessity to aliquot specimens prior to testing.

Herpes simplex virus type 1 (HSV-1) and HSV-2 are common causes of disease worldwide, with transmission resulting from direct contact with virus-infected secretions. The prevalence of HSV-1 infection increases with age, and >70% of adults worldwide are seropositive for the virus (18). The incidence of antibodies to HSV-2 is dependent on age, sex, and risk factors (e.g., number of sexual partners) and may reach 60 to 95% in certain high-risk groups, such as patients infected with HIV (11, 13, 20). However, a relatively small percentage of patients (10 to 20%) know that they are infected with genital herpes (12, 13, 21), thereby contributing to increased transmission of disease.

The diagnosis of HSV-associated disease is routinely made based on clinical findings and supported by laboratory testing using PCR or viral culture (13, 22). However, in instances of subclinical or unrecognized HSV infection, serologic testing for immunoglobulin G (IgG) antibodies to type-specific HSV glycoprotein G (gG) may be useful. Due to significant antigenic cross-reactivity among HSV structural proteins, only gG-based serologic assays have been shown to accurately differentiate between IgG class antibodies to HSV-1 and HSV-2 (2, 4, 9, 15), and FDA-approved, conventional, HSV type-specific enzyme immunoassays (EIA) and Western blot (WB) assays are commercially available. Although EIA and WB have demonstrated excellent sensitivity and specificity (2, 3, 15, 24), they require separate assays to be performed for the detection and differentiation of antibodies to HSV-1 and HSV-2. This may increase the potential for aliquoting errors, as well as the associated technologist and instrument time required for testing.

Recently, a number of multiplex flow immunoassays (MFI) have been described for the serologic evaluation of various infectious diseases (6, 8, 16). This approach is similar to traditional EIA but allows for the simultaneous detection and identification of multiple analytes in a single reaction tube. MFI technology uses a liquid suspension array of up to 100 unique microspheres (5- to 6-μm beads), each conjugated to a different capture molecule (e.g., antibody, antigen, or 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. Since 2008, three multiplex flow immunoassays (AtheNA Multi-Lyte [Zeus Scientific], BioPlex 2200 [Bio-Rad Laboratories], and Plexus HerpeSelect [Focus Diagnostics]) have received FDA clearance for the detection and differentiation of IgG class antibodies to HSV-1 and HSV-2. These assays are fully automated and designed for high-throughput analysis of the HSV type-specific antibody response.

Due to increasing test volumes (~105% in the past 3 years) and the limitations of conventional methods for HSV antibody
testing (e.g., limited throughput and labor-intensive testing), we undertook a study to evaluate and compare the AtheNA, BioPlex, and Plexus multiplex assays for the detection and differentiation of IgG class antibodies to HSV-1 and HSV-2. The objective of this study was to compare the results of MFI and EIA testing, using WB to further evaluate specimens showing discordant results.

MATERIALS AND METHODS

Study design. Serum specimens (n = 505) submitted to our reference laboratory for routine gG type-specific HSV IgG antibody testing by EIA (HerpeSelect; Focus Diagnostics, Cypress, CA) were also tested by the AtheNA Multi-Lyte (Zeus Scientific, Raritan, NJ), BioPlex 2200 (Bio-Rad Laboratories, Hercules, CA), and Plexus HerpeSelect (Focus Diagnostics) multiplex flow immunoassays. Specimens showing discordant results after initial testing were repeated by EIA and all three multiplex assays, with further discrepancies being evaluated by gG type-specific WB (University of Washington, Seattle, WA). The study protocol was reviewed and approved by the institutional review board at the Mayo Clinic.

Enzyme immunoassay. Testing by EIA was performed according to the manufacturer’s instructions, using the HerpeSelect HSV-1 and HSV-2 IgG EIA (Focus Diagnostics). The HerpeSelect EIA uses microwells coated with recombinant gG-1 (molecular mass, 35 to 45 kDa) or gG-2 (80 to 110 kDa) antigens. Testing was completed on a Triturus automated EIA analyzer (Grifols S.A., Barcelona, Spain).

Multiplex flow immunoassays. In addition to testing by EIA, each specimen was tested by the AtheNA, BioPlex 2200, and Plexus HSV-1 and HSV-2 IgG multiplex assays according to the manufacturers’ instructions. The positive and negative controls provided with each kit were included for quality control purposes. The principle of MFI technology has been reviewed previously (17, 23). In brief, the Athena Multi-Lyte HSV-1 and HSV-2 IgG Plus (Zeus Scientific) assay was performed on an automated immunoassay multiplexing system (AIMS) instrument (Inverness Medical, Princeton, NJ). The AtheNA assay uses an Luminex 100 automated analyzer (Bio-Rad Laboratories) and utilizes two different bead sets for the detection of IgG class antibodies to HSV-1 and HSV-2. These bead sets are coated with recombinant gG-1 (45 kDa) or synthetic gG-2 (70 kDa) antigen. For quality control purposes, the BioPlex assay also monitors the signals from three control bead sets incorporated into each reaction mixture. These internal controls verify the addition of patient sample to the reaction mixture, the absence of nonspecific binding, and the performance of the detector. The data are initially calculated as the relative fluorescence intensity (RFI) and are then converted to a fluorescence ratio (FR), using an internal standard bead. The FR is compared to an assay-specific calibration curve to determine the analyte concentration in antibody index (AI) units. Results are then classified, according to their AI values, as negative (<0.9), equivocal (0.9 to 1.0), or positive (>1.0).

Testing by the Plexus HerpeSelect assay was performed on a Janus (Perkin Elmer, Waltham, MA) liquid handling system. The Plexus assay uses two distinct HSV antigen microspheres coated with recombinant gG-1 (35 to 45 kDa) or gG-2 (80 to 110 kDa) antigens. Two types of process control beads are also included in the bead suspension; these serve to verify the addition of serum to the reaction mixture and to monitor for nonspecific binding. Following incubation and wash steps, the mean fluorescence intensity from each HSV antigen bead is measured by a Luminex 100 system (Luminex Co., Austin, TX) and compared against a cutoff, and calculations are performed to categorize results as negative (<0.9), equivocal (0.9 to 1.1), or positive (>1.10).

WB. Specimens showing discordant results after repeat testing by EIA and MFI were analyzed by WB. Testing by WB was blinned to the results of EIA and MFI and was performed and interpreted at the University of Washington Virology Laboratory as previously described (5). Results were reported as positive, negative, or indeterminate (atypical reactivity) for antibodies to HSV-1 and/or HSV-2.

Timing and reproducibility studies. The average assay time for testing with each multiplex system was obtained by timing two separate runs (both 90 and 180 specimens/run) from the time the samples were placed on the instrument until the final results of the run were calculated. Intra-assay reproducibility for both HSV-1 and HSV-2 results were performed by testing a single positive sample 20 times on each platform. Interassay reproducibility studies were completed by analyzing five samples (three positive and two negative) over three separate runs for both HSV-1 and HSV-2. In addition to analyzing the agreement of qualitative results (positive or negative), the mean index value, standard deviation (SD), and percent coefficient of variation (CV) were also determined.

Statistics. Statistical analyses were performed using JMP software, version 7 (SAS Institute, Inc., Cary, NC). In addition to percent agreement, kappa coefficients were calculated as a secondary measure of agreement. Result agreement by kappa values was categorized as near perfect (0.81 to 1.0), substantial (0.61 to 0.8), moderate (0.41 to 0.6), fair (0.21 to 0.4), slight (0 to 0.2), or poor (<0) (14).

Equivalo results were considered negative for sensitivity calculations and positive for specificity calculations.

RESULTS

Agreement between EIA and MFI. To measure agreement, the results of EIA and MFI were compared following testing of 505 serum specimens. For HSV-1 IgG, the AtheNA, BioPlex, and Plexus assays demonstrated agreements of 94.9% (479/505 specimens), 97.8% (494/505 specimens), and 97.4% (492/505 specimens), respectively, with the results of EIA (Table 1). Kappa coefficients showed near-perfect agreement between EIA and the AtheNA (κ = 0.9), BioPlex (κ = 0.96), and Plexus (κ = 0.95) HSV-1 assays (Table 1). Specimens showing discordant results after repeat testing were further analyzed by WB. Among these samples, WB resolved 10/16 (62.5%), 5/7 (71.4%), and 1/5 (20.0%) samples in favor of the AtheNA, BioPlex, and Plexus HSV-1 assays, respectively. Following WB analysis, the adjusted overall agreements of the AtheNA, BioPlex, and Plexus HSV-1 assays were 96.8% (489/505 specimens), 98.8% (499/505 specimens), and 97.6% (493/505 specimens), respectively.

For HSV-2 IgG, the AtheNA, BioPlex, and Plexus assays showed agreements of 87.9% (444/505 specimens), 97.2% (491/505 specimens), and 96.8% (489/505 specimens), respectively, with EIA. Kappa coefficients showed substantial agreement between EIA and the AtheNA (κ = 0.72) assay and near-perfect agreement for the BioPlex (κ = 0.93) and Plexus (κ = 0.91) HSV-2 assays (Table 2). For specimens with discrepant HSV-2 IgG results, WB resolved 3/35 (8.6%), 3/10 (30.0%), and 2/7 (28.6%) samples in favor of the AtheNA, BioPlex, and Plexus HSV-2 assays, respectively, resulting in adjusted agreements of 88.5% (447/505 specimens), 97.8% (494/505 specimens), and 97.2% (491/505 specimens), respectively.

Timing and reproducibility studies. Timing studies showed that the AtheNA, BioPlex, and Plexus assays could provide complete analysis of 90 serum specimens in 3.1, 1.5, and 2.9 h, respectively, versus 3.1 h by EIA. When the run size was increased to 180 samples, the AtheNA, BioPlex, and Plexus assays showed average assay times of 6.1, 2.6, and 4.7 h, respectively, versus 5.2 h by EIA.

Intra-assay reproducibility studies showed 100% agreement for qualitative results (positive or negative) among the 20 replicates tested by the AtheNA, BioPlex, and Plexus assays, with coefficients of variation of 6.1, 4.1, and 5.3% for HSV-1 and of 6.5, 4.8, and 6.9% for HSV-2, respectively. In addition, interassay precision studies showed 100% agreement for qualitative results over three separate runs for each platform.
DISCUSSION

It has been proposed that the recognition of persons infected with HSV through the use of gG-based, type-specific IgG serology may reduce the incidence of neonatal herpes and transmission of genital disease (7, 10). However, there are concerns that increased serologic screening for HSV may lead to overtreatment with antivirals, costly medical intervention, and unnecessary patient anxiety. These concerns are partially due to the limitations of current HSV serologic tests, including the inability to predict the risk of transmission, distinguish active from latent disease, or discriminate among anatomical sites of infection (e.g., oral versus genital) (1, 25).

Despite these limitations, clinical laboratories are experiencing a significant increase in the number of specimens submitted for HSV serologic testing. In 2008, our reference laboratory received 70,164 serum specimens (140,328 total tests) for HSV type-specific IgG serology. This represented a 26% increase in specimen volume compared to the previous year. These figures suggest a need for testing platforms which allow for a rapid, high-throughput analysis of the HSV serologic response.

### TABLE 1. Comparison of three multiplex flow immunoassays to EIA for the detection of IgG class antibodies to HSV-1 in prospective serum specimens (n = 505)

| Assay and result | No. of specimens with HerpeSelect HSV-1 IgG EIA result | % Sensitivity (95% CI) | % Specificity (95% CI) | % Agreement (95% CI) | Kappa coefficient |
|------------------|----------------------------------------------------|-----------------------|------------------------|----------------------|------------------|
|                  | Positive | Negative | Equivocal | Positive | Negative | Equivocal | Positive | Negative | Equivocal | Positive | Negative | Equivocal | Positive | Negative | Equivocal |
| AtheNA HSV-1 IgG |          |          |          | 254     | 15       | 0        | 99.2 (97.0, 99.9) | 90.7 (86.4, 93.8) | 94.9 (92.5, 96.5) | 0.90 |
| Positive         |          |          |          | 1       | 225      | 1        | 90.7 (86.4, 93.8) | 94.9 (92.5, 96.5) | 0.90 |
| Negative         |          |          |          | 1       | 8        | 0        | 90.7 (86.4, 93.8) | 94.9 (92.5, 96.5) | 0.90 |
| BioPlex HSV-1 IgG|          |          |          | 254     | 5        | 0        | 99.2 (97.0, 99.9) | 96.8 (93.7, 98.5) | 97.8 (96.1, 98.8) | 0.96 |
| Positive         |          |          |          | 2       | 240      | 1        | 96.8 (93.7, 98.5) | 97.8 (96.1, 98.8) | 0.96 |
| Negative         |          |          |          | 0       | 3        | 0        | 96.8 (93.7, 98.5) | 97.8 (96.1, 98.8) | 0.96 |
| Equivocal        |          |          |          |          |          |          | 96.8 (93.7, 98.5) | 97.8 (96.1, 98.8) | 0.96 |
| Plexus HSV-1 IgG |          |          |          | 247     | 3        | 0        | 96.5 (93.4, 98.2) | 98.8 (96.3, 99.8) | 97.4 (95.6, 98.5) | 0.95 |
| Positive         |          |          |          | 2       | 245      | 1        | 98.8 (96.3, 99.8) | 97.4 (95.6, 98.5) | 0.95 |
| Negative         |          |          |          | 0       | 0        | 0        | 98.8 (96.3, 99.8) | 97.4 (95.6, 98.5) | 0.95 |
| Equivocal        |          |          |          |          |          |          | 98.8 (96.3, 99.8) | 97.4 (95.6, 98.5) | 0.95 |

* Ten of these 15 specimens were positive by WB; 2 of these 15 specimens were equivocal by WB.
* This specimen was positive by WB.
* All five of these specimens were positive by WB.
* Both of these specimens were positive by WB.
* One of these three specimens was positive by WB.
* Both of these specimens were positive by WB.

### TABLE 2. Comparison of three multiplex flow immunoassays to EIA for the detection of IgG class antibodies to HSV-2 in prospective serum specimens (n = 505)

| Assay and result | No. of specimens with HerpeSelect HSV-2 IgG EIA result | % Sensitivity (95% CI) | % Specificity (95% CI) | % Agreement (95% CI) | Kappa coefficient |
|------------------|----------------------------------------------------|-----------------------|------------------------|----------------------|------------------|
|                  | Positive | Negative | Equivocal | Positive | Negative | Equivocal | Positive | Negative | Equivocal | Positive | Negative | Equivocal |
| AtheNA HSV-2 IgG |          |          |          | 114     | 34       | 2        | 97.4 (92.4, 99.5) | 85.5 (81.6, 88.7) | 87.9 (84.8, 90.5) | 0.72 |
| Positive         |          |          |          | 1       | 330      | 0        | 97.4 (92.4, 99.5) | 85.5 (81.6, 88.7) | 87.9 (84.8, 90.5) | 0.72 |
| Negative         |          |          |          | 2       | 22       | 0        | 97.4 (92.4, 99.5) | 85.5 (81.6, 88.7) | 87.9 (84.8, 90.5) | 0.72 |
| BioPlex HSV-2 IgG|          |          |          | 115     | 9        | 2        | 98.3 (93.6, 99.9) | 97.4 (95.2, 98.7) | 97.2 (95.4, 98.4) | 0.93 |
| Positive         |          |          |          | 1       | 376      | 0        | 98.3 (93.6, 99.9) | 97.4 (95.2, 98.7) | 97.2 (95.4, 98.4) | 0.93 |
| Negative         |          |          |          | 0       | 1        | 0        | 98.3 (93.6, 99.9) | 97.4 (95.2, 98.7) | 97.2 (95.4, 98.4) | 0.93 |
| Equivocal        |          |          |          |          |          |          | 98.3 (93.6, 99.9) | 97.4 (95.2, 98.7) | 97.2 (95.4, 98.4) | 0.93 |
| Plexus HSV-2 IgG |          |          |          | 109     | 2        | 1        | 93.2 (86.9, 96.7) | 98.4 (96.6, 99.4) | 96.8 (94.9, 98.1) | 0.91 |
| Positive         |          |          |          | 5       | 380      | 1        | 93.2 (86.9, 96.7) | 98.4 (96.6, 99.4) | 96.8 (94.9, 98.1) | 0.91 |
| Negative         |          |          |          | 3       | 4        | 0        | 93.2 (86.9, 96.7) | 98.4 (96.6, 99.4) | 96.8 (94.9, 98.1) | 0.91 |

* Two of these 34 specimens were positive by WB; 3 of these 34 specimens were equivocal by WB.
* This specimen was negative by WB.
* Two of these nine specimens were positive by WB; two of these nine specimens were equivocal by WB.
* This specimen was negative by WB.
* One of two specimens was equivocal by WB.
* Three of these five specimens were positive by WB.
The results of this study showed that the AtheNA, BioPlex, and Plexus HSV-1 IgG assays demonstrate near-perfect agreement (κ > 0.81) with conventional testing by EIA. In addition, kappa coefficients showed substantial agreement between EIA and the AtheNA (κ = 0.72) HSV-2 IgG assay and near-perfect agreement for the BioPlex (κ = 0.93) and Plexus (κ = 0.91) assays (Table 2). A recent study by Martins et al. (16) evaluated the AtheNA HSV-1 and HSV-2 multiplex assay, using 332 serum specimens, with the results showing agreements of 93.4% (310/332 specimens) and 94.9% (315/332 specimens), respectively, with testing by HerpeSelect EIA (16). These results are similar to our findings, although our study showed a slightly lower overall agreement (87.9%) for the AtheNA HSV-2 IgG assay compared to EIA. This difference may be due to the larger sample size tested in our study or, potentially, to lot-to-lot variation in assay reagents used between the two studies. Interestingly, the numerical value (AU/ml) for 10/34 (29.4%) AtheNA HSV-2 IgG-positive, EIA-negative samples in our study was within 20% of the AtheNA assay cutoff. Further testing of these specimens by WB showed that 5/34 specimens (14.7%) were positive (n = 2) or equivocal (n = 3) for IgG class antibodies to HSV-2 (Table 2).

This study has several limitations. First, the precision studies were performed on a single instrument per platform, so potential interinstrument and interlaboratory variations were not evaluated. Second, the specimens included in this study were submitted to our reference laboratory without accompanying clinical information, so a comparison of results to other clinical or laboratory data was not possible. Therefore, the sensitivity and specificity data (Tables 1 and 2) were calculated by comparing the results of MFI directly to those of HerpeSelect EIA, using WB to further evaluate discrepant samples. Although WB has long been considered the gold standard method for HSV type-specific antibody detection, the HerpeSelect EIA has demonstrated comparable performance to that of WB (3, 4, 19), is FDA approved, and has the capacity for automation and high-throughput analysis. In future studies, it will be interesting to evaluate the performances of MFIs by using clinically defined cases. These additional studies will serve to further assess the diagnostic sensitivity and specificity of these assays and will provide valuable information on the role of this technology in serologic screening programs for HSV-1 and HSV-2.

In conclusion, we have demonstrated that the AtheNA, BioPlex, and Plexus multiplex immunoassays show high overall agreement with routine testing by EIA, while offering several advantages. First, each assay has the capability to assess the type-specific IgG class antibody response to both HSV-1 and HSV-2 in a single reaction tube. This may reduce technologist time, instrument time, and aliquot errors. Second, each MFI platform incorporates internal controls into the reaction mixture, thereby enhancing quality control and the efficiency of monitoring result accuracy. Third, intra- and interassay reproducibility studies showed excellent precision for each system, with 100% agreement for qualitative results among runs. Finally, testing by MFI may allow for a more rapid and high-throughput analysis of the HSV serologic response. Our data showed that testing on the BioPlex instrument resulted in a >50% reduction in turnaround time compared to that for EIA (1.5 h [BioPlex] versus 3.1 h [EIA] for 90 specimens). The results of this study demonstrate that the evaluated systems may prove beneficial for high-volume clinical laboratories experiencing significant increases in specimens submitted for HSV serologic testing.

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