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Evaluation of a Multiplexed Bead Assay for Assessment of Epstein-Barr Virus Immunologic Status

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Currently, serological assays using either indirect immunofluorescence assay or enzyme-linked immunosorbent assay (ELISA) are performed to evaluate the status of Epstein-Barr virus (EBV) infection in humans. Although these methods are reliable, they are limited to testing an antibody response to a single viral antigen per reaction, thus necessitating a panel of assays to complete the evaluation. In contrast, a new bead-based method (BioPlex 2200; Bio-Rad Laboratories, Hercules, Calif.) can analyze the humoral response to multiple antigens in a single tube. This approach potentially reduces overall cost, turnaround time, and sample volume. The aim of this study was to evaluate the multiplexed EBV serologic assays performed on the BioPlex 2200 platform compared to results of conventional heterophile and ELISA-based assays. A total of 167 nonconsecutive, stored serum samples from adult and pediatric patients submitted for EBV serologic studies were used in the evaluation. Concordance between results generated by the BioPlex 2200 system and conventional assays was calculated. The anti-EA-D assay had the lowest concordance at 91%. The BioPlex 2200 system showed 97% agreement with conventional heterophile and anti-nuclear antigen assays and 92% agreement with the anti-VCA IgG and immunoglobulin M assays. Agreement between the BioPlex 2200 system and conventional testing was 92% with respect to categorization of acute versus nonacute EBV disease. The correlation between these two systems with regard to assignment into one of four categories of EBV status was also good (82%). In summary, there is excellent correlation between contemporary EBV serologic testing and the BioPlex 2200 system.

Epstein-Barr virus (EBV), a member of the family of human herpesviruses, infects up to 95% of adults worldwide (13). In most populations, EBV infection is generally established during childhood, usually with little or no sequelae (12). However, infection in an adolescent or adult patient not exposed to the virus in early childhood often leads to infectious mononucleosis, the most severe acute form of EBV-related disease. In addition, evidence of EBV infection has been demonstrated in patients with a wide variety of benign and malignant lesions, including Burkitt's lymphoma and nasopharyngeal carcinoma, and has been associated with the development of B-cell lymphoproliferative disease in patients who are post-solid organ or hematopoietic stem cell transplantation (10).

The diagnosis of acute EBV infection can be challenging since the symptoms are typically protean, resembling those of other viral infections. Although the presence of atypical lymphocytes on a peripheral smear supports the diagnosis of infectious mononucleosis, these are not universally detectable. Furthermore, because of the impracticality of EBV culture and the high prevalence of EBV in the population, specific viral detection by culture or PCR is not frequently used. Alternatively, the detection of heterophile antibodies and specific antibodies against EBV antigens has proven useful in the diagnosis of acute EBV infection, especially in cases with a high index of suspicion and a lack of atypical lymphocytes.

There are four basic categories of EBV status into which patients can be divided by serology. These include patients

with no history of infection, patients who are acutely infected, patients with evidence of a prior or remote infection, and patients with reactivation of latent virus (14). Anti-EBV serology, in combination with heterophile antibody testing, has been used to categorize patients in this manner, and the classification of patients based on the pattern of antibody responses has been reviewed elsewhere (9, 13, 14). Briefly, acute disease is suggested when immunoglobulin M (IgM) antibodies to the viral capsid antigen (VCA) are detected. The presence of heterophile antibodies and/or IgG antibodies to the D component of the EBV early antigen (EA-D) further supports the diagnosis of acute infection. In patients with a prior or remote infection, anti-VCA IgG and IgG antibodies to the EBV nuclear antigen (NA) are typically observed. The anti-VCA IgG antibodies slowly rise during acute infection but persist for life. In contrast, the anti-VCA IgM and anti-EA-D IgG antibodies rapidly decline as the patient recovers and are typically undetectable after 12 months (8, 13, 14). Patients who have reactivated latent virus will likely have evidence of past infection, e.g., anti-VCA IgG and anti-NA IgG, but also one or more markers of acute infection such as anti-VCA IgM. Because patients recovering from a recent acute infection might have antibody profiles similar to those with reactivated virus, it is sometimes difficult to distinguish these two groups of patients based on serology alone. In addition to patients who present with a possible acute infection, EBV serology is often performed on patients being evaluated for solid-organ or bone marrow transplant (10).

At present, the humoral response to EBV infection is commonly evaluated using indirect immunofluorescence assay (IFA) or enzyme-linked immunosorbent assay (ELISA)-based formats. According to a 2003 survey of laboratories accredited

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by the College of American Pathologists, ELISA-based methods are now the most widely used (2). Furthermore, ELISA methods are well characterized and are considered to be the reference laboratory methods (3, 5, 7, 14, 15). Recently, Bio-Rad Laboratories (Hercules, Calif.) introduced a new method for the detection of antibodies to EBV (BioPlex 2200). The instrument combines Lab-Map multi-analyte profiling technology (Luminex, Austin, Tex.) (6) with unique antigen-coated fluoromagnetic bead chemistry and versatile software. Briefly, dyed (fluorescent) bead sets, each of which is coated with a different, specific EBV antigen, are mixed with diluted patient sample. After incubation and a wash cycle, an anti-human IgG or IgM antibody conjugated with phycoerythrin (PE) is added. The instrument can then identify beads based on the fluorescence of the dyes and quantitate antibody on each bead based on the fluorescence of the PE. This is a robust system that can assess the response to multiple, independent antigens in a single incubation (multiplex). It has a number of potential advantages over conventional microtiter plate serologic assays, including a fully automated random access platform with minimal user interaction. Furthermore, common test groupings, such as the assays performed to evaluate EBV serologic status or ToRCH testing, can be performed in one tube. This strategy can potentially lower cost, minimize aliquot errors, and reduce turnaround time and sample requirements (5 μ l).

In the present study, the EBV assays on the BioPlex 2200 system were evaluated by comparing them to a conventional rapid card assay for the detection of heterophile antibodies and ELISA-based assays for the detection of anti-VCA IgG and IgM, anti-NA IgG, and anti-EA-D IgG antibodies. In addition, the agreement between the two systems in categorizing patients by EBV status was assessed.

(These findings were presented in part at the American Association of Clinical Chemistry Annual Meeting in July 2004.)

MATERIALS AND METHODS

Patient samples. A total of 167 leftover serum samples sent to the laboratory for physician-ordered EBV testing between October 2002 and September 2003 were evaluated. The sample population was designed to include a mix of adults and children (<21 years of age), as well as a mix of transplant patients and individuals being evaluated for acute EBV infections. Twenty-three of the patients were selected because of positive heterophile and/or positive anti-VCA IgM serology in order to increase the possible number of patients with acute EBV disease. The present study was approved by the Washington University Institutional Review Board for Clinical Studies.

EBV testing. Captia (Trinity Biotech, Jamestown, N.Y.) EBV anti-VCA IgG and Wampole (Wampole Laboratories, Cranbury, N.J.) EBV anti-VCA IgM, anti-EA IgG, and anti-NA IgG analyses were performed as described in the manufacturers' package inserts on the Dynex DSX (Dynex Laboratories, Chantilly, Va.) automated platform. Briefly, diluted patient serum was incubated (30 min for VCA-IgM and 20 min for EA-IgG, NA-IgG, and VCA-IgG) with antigen-bound microtiter plates. Antigen-antibody complexes bound to the plate were then incubated with horseradish peroxidase-conjugated goat anti-human IgG or IgM (30 min for IgM and 20 min for other antibodies) and developed (10 min) with tetramethylbenzidine. The reactions were stopped with H₂SO₄, and color was spectrophotometrically detected at 450 nm. Samples used for IgM detection were treated to remove excess IgG with goat or sheep anti-human IgG. The antigen for the IgM anti-VCA kit is affinity-purified VCA (gp125). The antigen for IgG anti-VCA is purified recombinant VCA (47-kDa fusion protein including 53 amino acids from the C terminus of p18). EA-D antigen is a recombinant protein derived from the carboxy terminus of the EA-D gene, representing 200 codons. NA-1 antigen is a recombinant protein derived from the carboxy terminus of the NA-1 gene, also representing ~200 codons.

The Status Mono (LifeSign LLC, Somerset, N.J.) heterophile antibody assay was performed as described in the manufacturer's package insert. Briefly, patient

serum was placed on the test card by using the supplied capillary tube. Two to three drops of developer solution (phosphate-buffered saline with 0.2% sodium azide) were then added to the same well, and the cartridge was allowed to develop for 8 min. The device contains a membrane strip coated with bovine erythrocyte extract (test lane) and a pad impregnated with the monoclonal mouse anti-human IgM antibody-dye conjugate in a protein matrix. A positive result was reported if a line appeared in the test lane and in the internal control lane. A single line in the control lane only was considered negative.

The five assays were also performed by using the Bio-Rad BioPlex 2200 system. These assays were carried out by the manufacturer. At that time, the assays were experimental and not yet available commercially. Market introduction after U.S. Food and Drug Administration clearance is expected in late 2004. The manufacturer was blinded to the ELISA and heterophile test results. Briefly, two sets of incubations were performed for each sample: one for the IgG assays and one for the IgM assays. Colored beads coated with the *Escherichia coli*-derived recombinant VCA (40-kDa), EA-D (28-kDa), or NA-1 (28- and 45-kDa) antigens were mixed together, along with the patient sample and diluent and then allowed to incubate at 37°C for 20 min. After a wash cycle, anti-human IgG antibody conjugated to PE was added to the dyed beads and allowed to incubate at 37°C for 10 min. After removal of excess conjugate, the bead mixture was passed through the detector that identifies the beads based on the fluorescence of the dyes. The amount of antibody bound to the bead was determined by the fluorescence of PE. Raw data were initially measured as the relative fluorescence intensity and then converted to the fluorescence ratio by using a predyed internal standard bead. A series of calibrators were analyzed with the patient samples to convert fluorescence ratio to international units per milliliter. The heterophile and VCA-IgM assays were performed in a separate incubation. In these IgM assays, IgG antibodies were absorbed from the sample during mixing with the beads. After this step, the subsequent procedure was the same as that for the IgG incubation, except for the use of anti-human IgM instead of anti-IgG. The two beads used in this incubation were coated with an *E. coli*-derived recombinant VCA antigen (40 kDa) or horse erythrocyte stromal extract (heterophile antigen) (4, 11). Two additional control beads were also included in all IgG and IgM incubations. A serum verification bead and a blank bead were added to verify the addition of serum to the reaction vessel and the absence of significant nonspecific bead binding, respectively.

RESULTS

The patient population consisted of 125 children less than 21 years of age (75%) and 42 adults (25%). Forty-two of the patients (9 children and 33 adults) were being evaluated prior to solid-organ or hematopoietic stem cell transplant. A total of 28 patients had a clinical presentation and laboratory values consistent with infectious mononucleosis. A total of 35 patients had no evidence of previous exposure to EBV, whereas 67 patients had evidence of a past infection.

The BioPlex 2200 system was evaluated by assessing concordance to standard ELISA and heterophile antibody assays. As seen in Table 1, there is strong concordance between the two systems for each test evaluated. The anti-EA IgG assay has the lowest concordance at 91%, whereas the anti-VCA-IgG and IgM assays both have concordances of 92%. The comparison methods agree 97% of the time on the heterophile antibody and anti-NA IgG assays.

The evaluation of EBV disease status does not rely on a single antibody test but on the pattern of results obtained and clinical presentation. Table 2 demonstrates a generally accepted algorithm for classifying patients into an EBV status via serologic profile (5, 13, 14). Using this table, each patient was assigned an EBV status based on results of the conventional tests. This diagnosis was compared to a diagnosis derived from Table 2 and results from the BioPlex 2200 testing.

As seen in Table 3, when the conventional and BioPlex 2200 methods are compared with respect to assignment of acute versus nonacute EBV disease, the concordance is excellent (92%). As shown in Table 4, the agreement between the two

TABLE 1. Correlation of anti-EBV serology between the BioPlex 2200 and conventional assay systems

Correlation (antibody and result) ^c	No. of sera that gave a conventional-assay result that was:			Concordance (%)
	Positive	Negative	Equivocal ^a	
BioPlex 2200 (anti-VCA IgG) vs Trinity ELISA				
Positive	102	2		92
Negative	11	52		
BioPlex 2200 (anti-VCA IgM) vs Wampole ELISA				
Positive	30	9		92
Negative	5	123		
BioPlex 2200 (anti-NA) vs Wampole ELISA				
Positive	85	3		97
Negative	2	77		
BioPlex 2200 (anti-EA) vs Wampole ELISA				
Positive	30	4	1	91
Negative	6	122	4	
BioPlex 2200 (heterophile antibody) vs Life Sign ^b				
Positive	26	2		97
Negative	3	136		

^a That is, borderline results in the Wampole anti-EA assay that could not be determined as positive or negative.
^b The Life Sign assay is a direct solid-phase immunoassay.
^c Result of assay with the BioPlex 2200 system.

systems with respect to assignment into one of four EBV serologic categories is also good (82%).

DISCUSSION

Although there is no true “gold standard” for the diagnosis of EBV-related disease, the evaluation of EBV serologic status is often performed on patients who present with symptoms consistent with infectious mononucleosis. In addition, patients who are to undergo a solid-organ or hematopoietic stem cell transplant are commonly tested due to the link between EBV and posttransplant lymphoproliferative disease (10). The conventional methods used to perform this testing are based on ELISA and IFA. Although these methods are well character-

TABLE 2. Patterns of anti-EBV antibody production associated with EBV disease stage^a

EBV disease stage	Production ^b of:				
	Anti-VCA IgG	Anti-VCA IgM	Anti-NA-1 IgG	Anti-EA-D IgG	Heterophile antibody
Susceptible	Neg	Neg	Neg	Neg	Neg
Primary acute	Pos/Neg	Pos	Neg	Pos	Pos/Neg
	Pos/Neg	Pos	Neg	Neg	Pos
	Pos	Neg	Neg	Pos	Pos
Prior or remote	Pos	Neg	Pos	Neg	Neg
	Pos	Pos/Neg	Pos	Pos/Neg	Pos/Neg
Reactivation or recovery	Pos	Pos	Neg	Neg	Neg

^a Information derived from references 5 and 14.
^b Pos, positive; Neg, negative; Pos/Neg, either positive or negative.

TABLE 3. Comparison of serological pattern interpretations in determining acute EBV infection between the BioPlex 2200 and conventional assay systems

Trinity/Wampole/LifeSign-based diagnosis	Bio-Rad multi-analyte bead system-based diagnosis (no. of sera)			Total
	Acute infection	No acute infection ^a	Unresolved ^b	
Acute infection	28	0	0	28
No acute infection	0	117	5	122
Unresolved ^b	1	8	8	17
Total	29	125	13	167 ^c

^a Includes cases of prior or remote infection and reactivation, recovery, and susceptible cases, as defined in Table 2.
^b Unresolved samples have patterns of antibody reactivity that are not consistent with any category listed in Table 2.
^c The overall concordance was calculated as (28 + 117 + 8)/167 = 92%.

ized and reliable, they require an aliquot of sample and reagents for each antigen tested. Since the patient sample has to be divided into aliquots multiple times, this can lead to so-called “pour-off” errors. Furthermore, the turnaround time and the cost of labor and materials rises proportionally with each test performed. In order to circumvent these practical issues, the idea of multiplexing EBV testing was developed (1). The EBV assays on the BioPlex 2200 system are performed in only two tubes, one for the IgG assays (EA, NA, and VCA-IgG) and one for the IgM assays (heterophile and VCA-IgM), allowing for all five tests to be performed with only two aliquots of reagents and patient sample.

As seen in Table 1, the agreement between the BioPlex 2200 and conventional assays for all five EBV assays was excellent (91 to 97%). In fact, the agreement is higher than that observed in another study comparing different ELISA and IFA methods (5). This was especially evident with anti-EA IgG, since the previous study compared ELISA to IFA methods, whereas the present study compares two solid-phase immunoassays.

Although each test has good concordance, each individual test disagreement could potentially lead to the assignment of a different EBV disease status. As such, the concordance between the two systems in assigning one of four specific EBV diagnoses was not as high (82%) as was observed for each individual test. Nevertheless, the agreement between these two systems in assigning a diagnosis is superior to that reported between IFA and ELISA systems in two similar studies (3, 5). As derived from Table 4, most of the disagreement in assigning EBV status occurs when one is determining whether a patient was previously infected or is in a reactivation or recovery stage. This was due to the slightly lower concordance in anti-VCA IgM and anti-EA-D IgG in this group of patients. This group of discordant patients was examined closely; we found no pattern with respect to the clinical presentation, to the results of the other EBV serologic tests, or to the results of additional serologic testing (such as with cytomegalovirus or herpes simplex virus).

The most common use of EBV serology is to help distinguish acute versus nonacute EBV disease, suggesting that this parameter is the most important in comparisons of the two assay systems. When the BioPlex 2200 system and conventional assays are compared on this basis (Table 3), they show excellent

TABLE 4. Comparison of clinical diagnosis based on serologic pattern from EBV testing on the BioPlex 2200 and conventional assay systems

Trinity/Wampole/LifeSign-based diagnosis	Bio-Rad multi-analyte bead system-based diagnosis (no. of sera)					Total
	Prior or remote infection	Primary acute infection	Reactivation or recovery	Susceptible	Unresolved ^a	
Prior or remote infection	57	0	8	1	1	67
Primary acute infection	0	28	0	0	0	28
Reactivation or recovery	7	0	11	0	2	20
Susceptible	0	0	0	33	2	35
Unresolved ^a	3	1	1	4	8	17
Total	67	29	20	38	13	167 ^b

^a Unresolved samples have patterns of antibody reactivity that are not consistent with any category listed in Table 2.

^b The over all concordance was calculated as $(57 + 28 + 11 + 33 + 8)/167 = 82\%$.

concordance (92%). Furthermore, the disagreements in Table 3 are primarily in distinguishing nonacute patients from those with unresolved serologic patterns. There is very little disagreement in distinguishing acute from either nonacute or unresolved patients. In fact, there was only one patient classified by either method as having an acute infection that was not classified as such by both methods. This patient had a bilateral lung transplant 1 year prior to testing and was on immunosuppressive therapy. She did not have classic infectious mononucleosis-like symptoms, nor did she have atypical lymphocytes on the peripheral smear. However, the EBV anti-VCA IgG and IgM and anti-EA-D IgG were positive by the BioPlex 2200 testing, but only the anti-VCA IgG and EA-D were positive by ELISA. There was no clear clinical evidence of infectious mononucleosis in this case, nor is there a gold standard that could be utilized to accurately diagnose EBV-related disease. As such, it was not possible to determine which of the two methods was correct with respect to the anti-VCA IgM results.

Most laboratories routinely offer only three of the five available EBV tests—the heterophile antibody test and the anti-VCA IgM and IgG assays—since most patients can be properly diagnosed by using these three antibodies. The assumptions typically made are that patients who are anti-VCA-IgM or heterophile positive have acute disease and those who are positive for IgG and negative for IgM and heterophile had a past infection but do not currently have disease. One problem with these assumptions is evident in Table 2. With only the three tests, patients who are VCA IgG and IgM positive and heterophile negative would be diagnosed with acute-phase illness. However, these patients could potentially be classified differently if all five tests were performed. For example, if a patient with the VCA and heterophile pattern described above were negative for both anti-NA-1 and anti-EA-D, then they would be considered to be in a recovery stage of the illness (last row of Table 2). This change in classification can have implications on long-term patient management, especially in neonatal and immunocompromised populations. Furthermore, if the anti-NA-1 was positive, then the illness would be more likely to be secondary to reactivation of latent virus, which could be useful information for physicians monitoring transplant patients (10). Ten patients within our population fit into one of these two scenarios. Since the practical issues limiting most centers to the three tests do not apply to the BioPlex 2200 system, all five tests can be routinely performed on each patient without an increase in labor, reagents, or sample requirements.

The BioPlex 2200 has been developed for high-throughput analysis of autoimmune analytes, as well as for common serological markers. The advantages of this system are that it offers full integration of sampling, antibody extraction, incubations, washing, and detection. The processing rate is 100 specimens/h (i.e., 300 EBV IgG test results or 200 EBV IgM/heterophile test result/h). The disadvantages are that it is a closed system, which limits its flexibility, and given the current menu, it is best suited for only larger laboratories.

This report provides a comparison between the Bio-Rad BioPlex 2200 and traditional ELISA-based assays. The two systems show excellent test-by-test concordance and also excellent agreement in the diagnosis of acute EBV infection. However, the BioPlex 2200 system offers practical advantages that allow for rapid evaluation of all five EBV antibodies. This can potentially yield laboratory benefits, as well as advantages in the medical management of patients that may have EBV infections.

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