Evaluation of Four Commercially Available Epstein-Barr Virus Enzyme Immunoassays with an Immunofluorescence Assay as the Reference Method

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Four commercially available enzyme immunoassays (EIAs) (Novitec, Biotest, Virotech, and DiaSorin) were evaluated, with an indirect immunofluorescence assay as the reference method, for Epstein-Barr virus (EBV) VCA (viral capsid antigen) immunoglobulin G (IgG), VCA IgM, or EBNA (EBV nuclear antigen) IgG at three different locations (Homburg, Stuttgart, and Dresden). Serum samples from 66 immunocompetent patients with infectious mononucleosis, 73 patients without prior EBV infection, and 96 patients with past EBV infections and 29 serum samples with possible cross-reactions to other herpesviruses were included. In addition, 25 samples from an extensively pretested panel that is commercially available (Boston Biomedica) were tested. Each sample was tested at only one location. The four EIAs varied considerably in performance. When analyzing for EBV diagnosis, the Novitec assay performed the best, with 4.9% discrepant diagnoses, followed by the Biotest, Virotech, and DiaSorin assays, with 6.8, 11.7, and 14.0% discrepant diagnoses, respectively. On the basis of single-parameter analysis, the Novitec assay also showed the lowest number of discrepant results, with 3.5%, compared with the Virotech, Biotest, and DiaSorin assays, which produced 5.4, 6.4, and 8.6% discrepant results, respectively. VCA assays using affinity-purified native antigens performed better than assays with recombinant or synthetic antigens. The synthetic EBNA-1s showed the lowest concordance with the reference compared to recombinant p72. Commercially available EBV EIAs differed considerably in performance; however, some proved to be reliable and convenient alternatives to the indirect immunofluorescence assay for routine diagnostics. Native antigens, rather than synthetic peptides, are favored for EBV serology testing.

Epstein-Barr virus (EBV), also classified as human herpesvirus 4, persists lifelong after primary infection. The virus can cause a wide variety of symptoms during a primary infection, depending on the host’s age, ranging from asymptomatic infection to severe infectious mononucleosis with serious complications. Other infections agents, such as cytomegalovirus (CMV), human immunodeficiency virus, rubella virus, or Toxoplasma gondii, may cause similar syndromes. Hematological malignancies are another important differential diagnosis. Thus, the key concern of EBV diagnostics is the distinction of a primary infection from seronegativity and past infection.

The diagnosis of a primary EBV infection builds upon an EBV-specific test for immunoglobulin G (IgG) and IgM antibodies to viral capsid antigens (VCA) and IgG antibodies to the EBV nuclear antigens (EBNA), especially EBNA-1, as the minimal requirement (10). Although the “gold standard” technique in EBV diagnostics is the indirect immunofluorescence assay (IFA), the enzyme immunoassay (EIA) technique is often used in routine diagnostics because of its reliability in high-throughput analyses.

EBV is reactivated frequently, resulting in intermittent excretion of the virus through saliva. Serological EBV reactivation has been studied in detail by using parameters such as antibodies to early antigens, VCA IgA, or the EBNA-1 IgG/EBNA-2 IgG ratio and different other parameters. However, since no clinically relevant disease is generally accepted as being linked to EBV reactivation in immunocompetent individuals, these parameters are only of limited value for the key concerns of routine EBV diagnostics. In contrast, in immunosuppressed patients, EBV reactivation plays a major role and is associated with disorders such as posttransplant lymphoproliferative disease or lymphoma in AIDS patients. However, serological diagnosis of EBV reactivation failed to correlate with the EBV viral load in immunosuppressed patients (8). Thus, antibody diagnostics is discouraged in these individuals.

The aim of the present study was to evaluate four commercially available EIAs, with IFA as the reference method, under routine conditions at three different locations. To address the key concerns of EBV, 264 samples from immunocompetent individuals, comprising EBV-seronegative and -seropositive subjects without EBV-related symptoms and patients with infectious mononucleosis, were tested. In addition, serum samples from patients with possible IgM cross-reactions to EBV due to acute CMV, herpes simplex virus (HSV), or varicella-zoster virus (VZV) disease (all IgM positive) were analyzed. A commercially available precharacterized EBV mixed-titer panel was assayed as well.

MATERIALS AND METHODS

Patients and samples. A total of 264 serum samples from immunocompetent individuals were tested. Sixty-six were from patients with infectious mononucle-
Four commercially available EIAs were evaluated with the IFA as the reference method. To address the key concern in EBV diagnostics, the distinction of a primary infection from seronegativity and a past infection, 66 serum samples from patients with clinically diagnosed infectious mononucleosis, 73 serum samples from individuals with no EBV infection, 96 serum samples from patients with previously acquired EBV infections without EBV-related symptoms, and 29 serum samples from patients with a possible cross-reaction to EBV due to CMV, HSV, or VZV disease (all IgM positive) were included. Finally, a panel of 25 commercially available and extensively pretested samples (the BBI panel) were analyzed together with the four EIAs and the IFA.

Each of the five assay systems analyzed three parameters: VCA IgG, VCA IgM, and EBNA-1 IgG. The results were interpreted in accordance with the criteria of the respective manufacturers (see Materials and Methods) and allowed a diagnosis of EBV primary infection, past infection, seronegative status, or indeterminate status, respectively. With the IFA as the reference method, discordant EBV diagnoses are summarized in Table 1.

The Novitec and Biotest assays matched the IFA best, showing only about 5% and 7% discordant results, respectively, whereas the Virotech and DiaSorin assays produced substantial 12% and 14% differences, respectively. The results obtained at the three locations were in good concordance and almost identical. The discrepant serum samples were mainly diagnosed as primary infections by the four EIAs. The Novitec and Biotest assays found around 80% primary infections. The Virotech assay found approximately 40% primary infections and, additionally, 40% with isolated EBNA-1 IgG—an implausible result. Among the discrepant serum samples, the DiaSorin...
TABLE 2. Single-parameter performance<sup>a</sup>

| Parameter and result | Novitec | Biotest | Virotech | DiaSorin |
|----------------------|---------|---------|----------|----------|
| VCA IgG              |         |         |          |          |
| False positive       | 3/73 (4.1) | 10/73 (13.7) | 2/73 (2.7) | 8/73 (11.0) |
| False negative       | 6/191 (3.1) | 4/191 (2.1) | 15/191 (7.9) | 7/191 (3.7) |
| VCA IgM              |         |         |          |          |
| False positive       | 5/203 (2.5) | 19/203 (9.4) | 12/203 (5.9) | 15/203 (7.4) |
| False negative       | 6/61 (9.8) | 11/61 (18.0) | 3/61 (4.9) | 11/61 (18.0) |
| EBNA-1               |         |         |          |          |
| False positive       | 2/139 (1.4) | 4/139 (2.9) | 4/139 (2.9) | 22/139 (15.8) |
| False negative       | 6/125 (4.8) | 3/125 (2.4) | 7/125 (5.6) | 5/125 (4.0) |
| Total                | 28/792 (3.5) | 51/792 (6.4) | 43/792 (5.4) | 68/792 (8.6) |

<sup>a</sup> Number of false-negative or false-positive results compared to the IFA reference method.

The assay detected 60% primary infections and 10% with isolated EBNA-1 IgG.

Not only the EBV diagnosis, based on the interpretation of all three parameters VCA IgG, VCA IgM, and EBNA-1 IgG, but also the performance of each individual parameter, is relevant when analyzing the quality of the four EIA systems. The serum samples were considered truly positive or truly negative according to clinical disease and IFA results; discrepant results are summarized in Table 2.

The best concordance with the IFA in single-parameter analysis was observed with the Novitec assay, followed by the Virotech, Biotest, and DiaSorin assays. Again, the results obtained at the three different locations were almost identical.

Simultaneous detection of VCA IgG, VCA IgM, and EBNA-1 IgG should be rare in EBV diagnostics and should be observed only after primary infection, when IgM persists for a while and EBNA-1 IgG is already produced, or during reactivations with increased IgM and EBNA-1 IgG not yet lost. Thus, the frequency of this marker combination is a good indicator of assay performance and the plausibility of the results obtained. The combination was not found by the IFA but was observed in 3 samples by the Novitec assay (1.1%), in 9 samples by the Biotest assay (3.4%), and in 19 serum samples by the DiaSorin and Biotest assays (7.2%)

The results obtained with the BBI panel are shown in Table 3. The samples were pretested by three assays for heterophilic antibodies, two EIAs, and two IFAs by the provider, BBI, but were not clinically characterized (see Materials and Methods). Results of the provider’s pretesting, rather than those of the in-house IFA performed at location A (Homburg), were used as the reference. Some reference results were discrepant (indices in Table 3) and thus not comparable. However, 65 were concordant. Of these, two were discrepant with respect to the Novitec assay (3.1%), three were discrepant with respect to the IFA (4.6%), four were discrepant with respect to the Virotech assay (6.2%), six were discrepant with respect to the Biotest assay (9.2%), and eight were discrepant with respect to the DiaSorin assay (12.3%). Thus, the results of the BBI panel confirmed the findings that the Novitec and Virotech assays are more reliable for single-parameter analysis.

DISCUSSION

Several assays are available and are routinely used for the diagnosis of EBV infection that employ various techniques but differ greatly in performance (3, 4, 6, 7, 12, 14, 15). The present study was conducted to compare four EIAs with the IFA as the gold standard reference method. All of the EIAs tested are frequently used and are well established in many laboratories. We aimed to compare the EIAs under routine conditions; samples were therefore tested once and not in duplicate.

The accurate distinction of a primary infection from a past infection or seronegative status is the key concern of EBV diagnostics. Reactivation of EBV resulting in virus replication is a common phenomenon but is normally clinically silent in immunocompetent individuals. In contrast, EBV reactivation causes considerable problems in immunosuppressed patients. However, serological methods are discouraged and PCR is favored for these patients (8). For this reason, serum samples from immunosuppressed individuals were excluded and we did not differentiate between past and reactivated infections. Accordingly, there was no need to test further parameters, such as antibodies to early antigen or VCA IgA.

The best concordance with the reference method for EBV diagnosis was obtained with the Novitec assay, followed by the Biotest assay. The Virotech and DiaSorin assays produced more than twice as many discrepant results as the Novitec assay.

TABLE 3. Discrepant and divergent results obtained with the BBI reference panel<sup>b</sup>

| Sample no. | BBI reference | VCA IgG/VCA IgM/EBNA IgG score |
|------------|---------------|--------------------------------|
|            | Novitec | Biotest | Virotech | DiaSorin | IFA |
| 1          | −/−/− | +/−/− | +/+/+ | +/+/+ | +/+/− |
| 2          | −/−/− | +/−/− | +/+/+ | +/+/+ | +/+/− |
| 3, 4, 5    | +/+/− | −/−/− | −/−/− | −/+/− | −/+/− |
| 6          | +/+/− | +/−/− | +/+/− | +/+/− | +/+/− |
| 7          | +/+/− | +/+/− | +/+/− | +/+/− | +/+/− |
| 8          | +/+/− | +/+/− | +/+/− | +/+/− | +/+/− |
| 9          | +/+/−/− | +/+/− | +/+/− | +/+/− | +/+/− |
| 10, 11     | +/+/−/− | +/+/− | +/+/− | +/+/− | +/+/− |
| 12         | +/+/+ | +/+/+ | +/+/+ | +/+/+ | +/+/+ |
| 13         | +/+/+ | +/+/+ | +/+/+ | +/+/+ | +/+/+ |
| 14         | +/+/+ | +/+/+ | +/+/+ | +/+/+ | +/+/+ |
| 15         | +/+/+ | +/+/+ | +/+/+ | +/+/+ | +/+/+ |
| 16         | +/+/+ | +/+/+ | +/+/+ | +/+/+ | +/+/+ |
| 17         | +/+/+ | +/+/+ | +/+/+ | +/+/+ | +/+/+ |
| 18, 19, 22 | +/+/+ | +/+/+ | +/+/+ | +/+/+ | +/+/+ |
| 20, 21     | +/+/+ | +/+/+ | +/+/+ | +/+/+ | +/+/+ |
| 22         | +/+/+ | +/+/+ | +/+/+ | +/+/+ | +/+/+ |
| 23         | +/+/+ | +/+/+ | +/+/+ | +/+/+ | +/+/+ |
| 24         | +/+/+ | +/+/+ | +/+/+ | +/+/+ | +/+/+ |
| 25         | +/+/+ | +/+/+ | +/+/+ | +/+/+ | +/+/+ |

<sup>a</sup> The BBI panel was pretested with three assays for heterophilic antibodies, two EIAs, and two IFAs (see Materials and Methods). If not otherwise indicated, samples were negative for heterophilic antibodies. Only discrepant results and all samples with divergent results within the reference assays (as indicated by indices) are shown. Discordant results are in bold. +, positive; −, negative; ±, divergent.

<sup>b</sup> The BII IFA produced a weak positive result with sample 5.

<sup>c</sup> All three assays for heterophilic antibodies yielded positive results.

<sup>d</sup> Zeus and BII IFA negative.

<sup>e</sup> Meridian EIA positive.

<sup>f</sup> False negative by Meridian VCA-IgG EIA since the respective Meridian assay was EBNA-1 IgG positive.

<sup>g</sup> Heterophilic antibodies indicated by weakly positive Seradyn assay result obtained with sample 22.

<sup>h</sup> Incstar EIA negative.
VCA of the Biotest assay produced poor results (Table 2). This purified native antigen (p125), which performed best. The full-length p23 and the carboxy-terminal half of p18). The whereas the Biotest assay makes use of recombinant VCAs assays. The DiaSorin assay uses a synthetic peptide (p18), which make use of the full-length EBNA-1 antigen, the Biotest assay employs a protein lacking the glycine-alanine repeat sequence that is known to occasionally cross-react with cellular proteins (11, 13). Nevertheless, the number of false-positive EBNA-1 IgG results obtained with the Biotest assay was not lower than that obtained with the Novitec or Virotech assay. Thus, deletion of the glycine-alanine repeat sequence did not significantly affect assay performance with this panel of serum samples.

A greater variety of different proteins are employed in VCA assays. The DiaSorin assay uses a synthetic peptide (p18), whereas the Biotest assay makes use of recombinant VCAs (full-length p23 and the carboxy-terminal half of p18). The Novitec and Virotech assays, by contrast, employ an affinity-purified native antigen (p125), which performed best. The VCA of the Biotest assay produced poor results (Table 2). This is underscored by a comparable study in which—a apart from a lower number of false-positive VCA IgM results—almost similar results were obtained (5). The lowest performance, however, was seen with the VCA protein p18 utilized by the DiaSorin assay. Antibodies against this antigen are produced late during primary infection (2). This is reflected by the fact that all seven of the false-negative VCA IgG serum samples tested with the DiaSorin assay were samples from primary infections.

Combined, the native antigens were obviously superior, in this study, to the recombinant antigens. Synthetic antigens, which lack natural conformational epitopes, showed the lowest concordance with the reference IFA.

Not only the quality of the individual parameters but also their interpretation is a critical factor in assay performance. This is illustrated by the fact that the Novitec and Virotech assays, which use similar antigens, produced roughly identical single-parameter analysis results (Table 2). However, differences in interpretation criteria led to substantially more misdiagnoses on the basis of the Virotech assay (11.7%) than on the basis of the Novitec assay (4.9%; Table 1). Again, the difference in the performance of the Biotest assay in EBV diagnosis versus the single-parameter analysis is explained by interpretation criteria, which are based mainly on the reasonably good Biotest assay EBNA-1 IgG and not on its problematic VCA IgM.

Furthermore, the interpretation schemes of the manufacturers allow diagnoses of EBV infection states that lack clinical equivalence. The only clinically relevant diagnoses in immunocompetent patients are primary infection as a cause of mononucleosis, past infection to exclude mononucleosis, and the absence of antibodies to also exclude EBV disease but show susceptibility. Interpretations such as “transient,” “convalescent,” and “recent” infections by the manufacturers are of little additional help. One may ask if a “recent,” “transient,” or “convalescent” infection is correlated with infectious mononucleosis or not. If so, these attributes should be taken to indicate a primary infection. If not, a past infection has to be considered. Taken together, these problems emphasize the urgent need for standardization of the interpretation criteria.

EIA technology offers the benefits of automated high-throughput analyses and objective results in routine diagnostics, whereas IFA technology is time consuming and takes experienced personnel for fluorescence image reading. Generally speaking, the IFA is less sensitive than the EIA. Nevertheless, the specificity of the IFA gold standard remains superior since unspecific reactions, such as anticytoplasmic reactivity, are unambiguously detected.

In summary, our results show that two of the four EIAs tested agree well with the reference IFA in the distinction of a primary infection from seronegativity and a past infection and may thus constitute convenient alternatives to the gold standard IFA.

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