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Evaluation of 4 methods for the serological diagnosis of Epstein–Barr virus infection using an immunofluorescence assay as the reference method

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Background/aim: Tests specific for VCA IgM, VCA IgG, and EBNA IgG are used to diagnose Epstein–Barr virus (EBV) infections and interpret disease status. The immunofluorescence assay (IFA) is accepted as the "gold standard" test. The purpose of this study was to evaluate the performance of 4 methods in comparison with IFA.

Materials and methods: In total, 101 serum samples were obtained from clinically suspected cases of EBV infection between May 2010 and May 2012 and evaluated by IFA. All serum samples were analyzed by an immunoblot assay, enzyme-linked fluorescent assay (ELFA), enzyme immunoassay (EIA), and immunochromatographic assay (ICA).

Results: ELFA and ICA results were in good agreement with IFA for the detection of VCA IgM, VCA IgG, and EBNA IgG. The results of the immunoblot assay agreed less well with IFA for EBNA IgG, while EIA results were not in agreement with IFA for EBNA IgG or VCA IgM.

Conclusion: Among the tests studied, ELFA and ICA appear to be suitable methods for the diagnosis and staging of EBV when considering cost-effectiveness, turnaround times, need for a specialist, and IFA concordance.

Key words: Epstein–Barr virus, ELISA, immunoblot assay, immunochromatographic assay

1. Introduction

The Epstein–Barr virus (EBV) is a member of the *Gammaherpesvirinae*, a subfamily of the family *Herpesviridae*. It is one of the most common human viruses and is distributed worldwide. More than 90% of the population becomes infected with EBV at some time during their life. The virus is acquired during childhood and usually does not cause any symptoms. However, approximately 10%–20% of adolescents and young adults in Western societies develop acute infectious mononucleosis. EBV is also associated with certain cancers, specifically Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's disease, and immunoblastic lymphoma (1,2).

Nonspecific and EBV-specific laboratory tests are used to diagnose EBV infection. The detection of heterophile antibodies, referred to as the Paul–Bunnell test, is a nonspecific test commonly used around the world for the diagnosis of EBV infection. However, this test produces false negative results in approximately 40% of children tested. Even higher percentages (92.9%) of false positive results are observed in young adults, although the sensitivity is higher in adults (2–4). In addition, heterophile antibodies are nonspecific and may also be present in non-EBV infections, malignancies, and autoimmune diseases (2,5). Negative results for the detection of heterophile antibodies are not evidence of the absence of EBV infection in children. Likewise, positive results do not always confirm the presence of acute EBV infection in these patients.

The detection of 3 analytes (VCA IgM, VCA IgG, and EBNA IgG) in combination is a specific test used for the interpretation of primary and past infection, and for the determination of the absence of EBV infection (6). A primary infection is characterized by the detection of VCA IgM and VCA IgG, with negativity for EBNA-1 IgG. Past infections are characterized by EBNA-1 IgG and VCA IgG detection, with negativity for VCA IgM. Evaluation of EA-D IgG may also be useful for determining the disease state when various serological patterns are encountered. Detection of EBNA-1 IgG excludes acute EBV infection, which is important during evaluation (2).

Several methods can be used for the serological diagnosis of EBV infection. Of these, the immunofluorescence assay (IFA) is accepted as the "gold standard" (7). However, standardization of this method is

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difficult due to its requirement for experienced personnel, the sometimes subjective interpretations, and time-consuming procedure. In this study, we evaluated the performance of 4 methods [immunoblot assay, enzyme-linked fluorescent assay (ELFA), enzyme immunoassay (EIA), and immunochromatographic assay (ICA)] in comparison with IFA.

2. Materials and methods

This study was approved by the local ethics committee.

2.1. Serum samples

In total, 101 serum samples obtained from clinically suspected cases of EBV infection between May 2010 and May 2012 were evaluated by IFA. The serum samples were stored at –20 °C following a diagnosis of EBV infection by IFA. Analysis of the samples using the 4 test methods was performed at room temperature without refreezing.

2.2. IFA

EBV-specific anti-VCA IgM, anti-VCA IgG, anti-EBNA IgG, and anti-EA IgG antibodies were assayed by the IFA test (Euroimmun, Germany) as a reference method. Patient samples were diluted 1:10 in PBS-Tween, and negative and positive controls were included in all tests. Control serum was added directly to the corresponding reaction field of the reagent tray. The tests were independently evaluated by 3 experienced specialists educated in this field.

2.3. Immunoblot assay

IgG and IgM antibodies against EBV VCA (gp125 and p19), EBV nuclear antigens (EBNA-1 and p22), and EA-D were analyzed using the Euroline anti-EBV profile 2 immunoblotting assay (Euroline, Euroimmun, Germany). Patient samples were diluted 1:51 and mixed well by vortexing.

2.4. ELFA

EBV VCA IgM, EBV VCA/EA IgG, and EBV EBNA IgG were detected using this assay, which contains p18 for VCA, p54 for EA, and p72 for EBNA (VIDAS, BioMérieux, France). The results are calculated automatically and expressed as an index of the ratio between the tested sample and a positive standard.

2.5. EIA

EBV-specific anti-VCA IgM, anti-VCA IgG, anti-EBNA IgG, and anti-EA IgG antibodies were detected using micro enzyme-linked immunosorbent assay (microELISA; Euroimmun, Germany). The samples were assayed at a 1:101 dilution. A calibration, negative control, and positive control were included in all tests.

2.6. ICA

EBV-specific anti-VCA IgM, anti-VCA IgG, and anti-EBNA IgG were investigated according to the manufacturer's instructions (VIRapid, Vircell, Spain). The results of the immunoblot assay, ELFA, EIA, and ICA were compared with that of the reference method (IFA).

2.7. Classification of disease state

The specimens were categorized as seronegative, primary infection, reactivation, or past infection based on the EBV-specific antibody profiles described by Klutts (8).

2.8. Statistical analysis

Statistical analyses were performed using SPSS 15 (SPSS Inc., USA). Kappa statistics were used to assess the agreement between tests, and the kappa values were evaluated according to Landis and Koch. Levels of agreement for the kappa value results were categorized as "almost perfect" (0.81 to 1.0), "substantial agreement" (0.61 to 0.80), "mostly in agreement" (0.41 to 0.60), and "poor to fair" (0 to 0.40) (9).

3. Results

Eleven primary infections, 1 past infection characterized by loss of EBNA, 60 past infections, 6 reactivations, 21 seronegative results, and 2 unknown results were obtained by IFA. Of the 11 serum samples diagnosed as primary infection by IFA, 10 were evaluated as primary infections by immunoblot assay, 6 by ELFA and ICA, and 3 by EIA. Fifty of the 60 serum samples diagnosed as past infections by IFA were evaluated as past infections by the immunoblot assay, 54 by ELFA, 47 by EIA, and 48 by ICA. Reactivations were not obtained by tests other than IFA (Table 1).

The agreement rate for anti-VCA IgM detection with respect to the reference test was 0.824 for immunoblot assay, 0.622 for ELFA, 0.076 for EIA, and 0.509 for ICA. These results indicate almost-perfect agreement between immunoblot assay and IFA for anti-VCA IgM detection, while the agreement classification for EIA was interpreted as "poor to fair". The agreement of ELFA and ICA with IFA was interpreted as "substantial agreement" and "mostly in agreement", respectively. The sensitivity was between 33.3% (EIA) and 91.7% (immunoblot assays), while the specificity was between 77.5% (EIA) and 96.6% (immunoblot assays) (Table 2).

An agreement rate of 0.653 was obtained for the immunoblot assay, 0.712 for ELFA, 0.734 for EIA, and 0.599 for ICA for anti-VCA IgG when compared with the reference test. While there was substantial agreement between the immunoblot assay, ELFA, EIA, and IFA for anti-VCA IgG, the agreement between ICA and IFA was interpreted as "mostly in agreement". The sensitivity was between 84.6% (ICA) and 97.4% (immunoblot assays), while the specificity was between 60.9% (immunoblot assays) and 87% (ELFA) (Table 3).

An agreement rate of 0.300 was obtained for the immunoblot assay, 0.568 for ELFA, 0.297 for EIA, and 0.590 for ICA for anti-EBNA IgG when compared with the reference test. While the results indicated that ELFA

Table 1. Comparison of assay interpretation.

Assay interpretation Primary infection		IFA					
		Primary infection	EBNA lost, past infection	Past infection	Reactivation	Unknown	Seronegative
Immunoblot-based assay	Primary infection	10	0	1	0	0	0
	EBNA lost, past infection	0	0	7	0	1	0
	Past infection	1	1	50	6	1	1
	Reactivation	0	0	0	0	0	0
	Unknown	0	0	0	0	0	6
	Seronegative	0	0	2	0	0	14
ELFA	Primary infection	6	1	0	0	0	0
	EBNA lost, past infection	0	0	0	0	0	0
	Past infection	4	0	54	4	2	1
	Reactivation	1	0	0	0	0	0
	Unknown	0	0	4	1	0	2
	Seronegative	0	0	2	1	0	18
EIA	Primary infection	3	0	11	2	0	0
	EBNA lost, past infection	0	0	0	0	0	0
	Past infection	8	1	47	4	2	1
	Reactivation	0	0	0	0	0	0
	Unknown	0	0	0	0	0	3
	Seronegative	0	0	2	0	0	17
ICA	Primary infection	6	1	1	1	0	1
	EBNA lost, past infection	0	0	0	0	0	0
	Past infection	3	0	48	3	2	1
	Reactivation	0	0	0	0	0	0
	Unknown	2	0	11	2	0	1
	Seronegative	0	0	0	0	0	18

Table 2. Agreement of assays for VCA IgM.

Assay interpretation Positive		IFA		Sensitivity (%)	Specificity (%)	Kappa
		Negative				
Immunoblot-based assay	Positive	11	3	91.7	96.6	0.824
	Negative	1	86			
ELFA	Positive	8	4	66.7	95.5	0.622
	Negative	4	85			
EIA	Positive	4	20	33.3	77.5	0.076
	Negative	8	69			
ICA	Positive	9	10	75	88.8	0.509
	Negative	3	79			

Table 3. Agreement of assays for VCA IgG.

Assay interpretation Positive		IFA		Sensitivity (%)	Specificity (%)	Kappa
		Negative				
Immunoblot-based assay	Positive	76	9	97.4	60.9	0.653
	Negative	2	14			
ELFA	Positive	70	3	89.7	87	0.712
	Negative	8	20			
EIA	Positive	75	6	96.2	74	0.734
	Negative	3	17			
ICA	Positive	66	4	84.6	82.7	0.599
	Negative	12	19			

and ICA mostly agreed with IFA for anti-EBNA IgG, the agreement of the immunoblot assay and EIA was interpreted as "poor to fair". The sensitivity was between 74.6% (EIA) and 89.8% (immunoblot assays), while the specificity was between 38.1% (immunoblot assays) and 73.8% (ICA) (Table 4).

The ELFA test required 40 min to determine the EBV status, while 190 min was required for IFA, 130 min for both the immunoblot assay and EIA, and 20 min for ICA. The immunoblot assay and ELFA were more expensive than the others. EIA and IFA required subjective comments by experienced personnel. Specialist equipment was needed for all tests investigated, with the exception of ICA.

4. Discussion

To determine the stage of EBV infection (acute, past, or reactivation), several parameters must be evaluated. When diagnostic tests for EBV infection are evaluated, the results are usually compared to the "gold standard" test, IFA. In this study, we evaluated the diagnostic performance of 2 immunoassays, an immunoblot assay, and an ICA by comparing them with IFA. We identified good correlations between the 4 methods and IFA for the detection of anti-VCA IgG. Detection of anti-VCA IgM by the immunoblot assay, ELFA, and ICA showed good correlation with IFA results. ELFA and ICA correlated well with IFA for anti-EBNA IgG detection. The agreement of ELFA, EIA, and ICA with IFA was interpreted as "poor to fair".

Table 4. Agreement of assays for EBNA IgG.

Assay interpretation Positive		IFA		Sensitivity (%)	Specificity (%)	Kappa
		Negative				
Immunoblot-based assay	Positive	53	26	89.8	38.1	0.300
	Negative	6	16			
ELFA	Positive	50	12	84.7	71.4	0.568
	Negative	9	30			
EIA	Positive	44	19	74.6	54.8	0.297
	Negative	15	23			
ICA	Positive	50	11	84.7	73.8	0.590
	Negative	9	31			

Immunoblot assays are considered to have high specificity for EBV serology; previous reports indicate that the results generally correlate well with IFA (7,10–12). Buisson et al. reported that immunoblot assays have diagnostic capabilities for EBV infection (7). Altuglu et al. reported an agreement between immunoblot assays and IFA for EBNA-1 IgG and anti-VCA IgM, but less agreement for anti-VCA IgG (10). Koidl et al. studied 60 IFA IgG- and IgM-positive samples with VIDAS, and reported 45 identical results (11). The additional western blot testing of the remaining 15 discrepant samples was reported to reveal 5 secondary reactivations, 6 past infections, 2 true primary infections, and 2 seronegative samples in their study (11). Sener et al. obtained satisfactory results using IFA, EIA, and western blotting for the serological diagnosis of EBV (12). The use of various recombinant antigens in line immune assays makes this method advantageous for detecting EBV status. However, in our study, reactivations were obtained only with the IFA test, and none of the other tests could identify these patients. This may be due to the relatively small number of samples. In this study, we compared the results of an immunoblot assay with those of the standard IFA method and obtained good agreement for the detection of anti-VCA IgG and anti-VCA IgM. However, there was less agreement between the immunoblot assay and IFA for the detection of anti-EBNA IgG.

ELFA is a widely used method for the diagnosis of EBV infection that can automatically detect anti-VCA IgM, anti-VCA/EA IgG, and anti-EBNA IgG. Numerous studies have assessed the performance of ELFA as a diagnostic method for infection. Comparisons between ELFA and IFA led Koidl et al. to report that ELFA may be an alternative to IFA testing, especially in high-throughput laboratories (11). To establish EBV profiles without using standard IFA measurements, Lupo et al. analyzed the results of ELFA and chemiluminescence assays and found that they performed similarly (13). We found a good correlation between ELFA and IFA for the detection of VCA IgG, VCA IgM, and EBNA IgG in this study. Of the 60 true past-infection cases, 54 were confirmed as past infections by ELFA, while 6 of 11 primary infections were confirmed as such by ELFA. These results may be due to the interpretation of IFA IgG avidity tests, which provide accurate information regarding disease state.

Enzyme immunoassays are relatively simple and rapid methods that are conventionally used for the diagnosis of EBV. Many studies have investigated the accuracy of EIA for the diagnosis of EBV infection. Rea et al. evaluated the acute and convalescent serological responses to EBV using the ELISA and IFA methods, and they reported ELISA to be a viable alternative to IFA (14). Feng et al. compared the results of anti-VCA IgM measurements using chemiluminescent immunoassay (CLIA) with those by ELISA in patients with infectious mononucleosis and primary EBV infection detected by IFA (15). They reported CLIA to be a more sensitive and specific method than ELISA for the diagnosis of infectious mononucleosis. Based on comparisons with IFA, Ory et al. reported that immunofiltration, CLIA, and ELISA may be useful for the diagnosis of EBV infections (16).

Devanthery et al. compared a bead-based assay with EIA and IFA and observed a good qualitative correlation among the 3 methods for detecting anti-VCA IgG and IgM (17). IFA displayed a considerably reduced sensitivity compared to the 2 other methods for the detection of anti-EBNA antibodies. Gartner et al. evaluated 4 commercially available EIAs using IFA as the reference method (18). They reported that 2 of the 4 EIAs agreed well with the reference IFA results for the distinction of a primary infection from seronegativity and a past infection, possibly constituting reasonable alternatives to the standard IFA method. It was also speculated that both the quality of the individual parameters and their interpretation are critical factors in assay performance. Our results indicate that EIA measurements correlated well with IFA for the detection of VCA IgG; however, both VCA IgM and EBNA IgG correlated with the IFA results only weakly.

The literature indicates that ICA has yet to be investigated as a diagnostic tool for EBV infection. To evaluate its effectiveness, we compared ICA with the standard procedure (IFA), and found it to be mostly in agreement. The ICA procedure is simple and does not require specialist equipment. The ease of use and the significantly higher sensitivity and specificity of ICA make it a good choice for the diagnostic testing of EBV infection.

Here we compared 4 methods that are used to detect antibodies against EBV (immunoblot assay, ELFA, EIA, and ICA) with the standard IFA method using a limited number of serum samples. Our results demonstrate that ELFA and ICA were in agreement with the standard IFA method for VCA IgM, VCA IgG, and EBNA IgG detection. There was less agreement between the results of IFA and those of both the immunoblot assay and EIA for EBNA IgG. The EIA results were also less in agreement with IFA for VCA IgM. After considering test cost-effectiveness, turnaround time, requirements for specialist equipment, and IFA concordance, we conclude that both ELFA and ICA may be suitable methods for the diagnosis and staging of EBV.

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