Application of a Commercial Immunoblot to Define EBV IgG Seroprofiles

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Background: Immunoblot (IB) techniques using different Epstein–Barr virus (EBV) antigens have been applied for detecting specific antibodies, making possible to obtain EBV seroprofiles in a single determination. The aim of this study was to evaluate a commercial IB for the detection of EBV-specific IgG (Euroimmun, Lübeck, Germany). **Methods:** A total of 117 samples classified as EBV primary recent infections ($n = 70$), past infections ($n = 29$), or not infected ($n = 18$) have been used. The samples were characterized by immunofluorescence, by testing EBV capsid antigens IgM and IgG (using indirect approaches) and EBV nuclear antigen (by anticomplement technique; Meridian Bioscience Inc.).

Key words: Epstein–Barr virus; immunoblot; immunofluorescence; infectious mononucleosis; seroprofiles

Results: Using the cut-off value as defined by the IB manufacturer, the concordance, relative sensitivity, and relative specificity were 85.5 (100/117), 94.3% (66/70), and 72.3% (34/47), respectively. If a corrected cut-off value was considered to classify the samples, the corresponding corrected figures were 89.7, 88.6, and 91.5%, respectively. **Conclusion:** Being a useful serological diagnostic tool, IB for testing EBV IgG seems to be an adequate approach to define EBV seroprofiles. However, efforts to better define the cut-off value should be made in order to improve the performance of the assay in evaluation. J. Clin. Lab. Anal. 29:47–51, 2015. © 2014 Wiley Periodicals, Inc.

INTRODUCTION

Infectious mononucleosis (IM) is a syndrome caused mainly by Epstein–Barr virus (EBV), although other infectious agents, including viruses (cytomegalovirus (CMV), human herpesvirus 6, human immunodeficiency virus, adenovirus, or herpes simplex virus), bacteria (*Streptococcus pyogenes*), and parasites (*Toxoplasma gondii*) (1), are identified as well as potential etiological agents. Of these, the most frequent one is CMV, responsible for more than 5% of IM cases (2, 3). EBV infections can be specifically diagnosed by serology, by detecting specific IgM against the viral capsid antigen (VCA). However, the high degree of cross-reactivity between EBV and CMV, as well as the polyclonal stimulation of memory lymphocytes elicited in the course of IM, reflected in positive IgM broad reactivity (4), makes the availability of confirmatory approaches important. The characterization of low-avidity VCA-specific IgG permits the confirmation of EBV primary infection (5). Furthermore, the estab-

lishment of antibody profiles is an adequate approach to achieve effective serological diagnosis of infection by EBV, since primary EBV infection is confirmed by the presence of VCA-specific IgG in the absence of EBV nuclear antigen (EBNA) antibodies (6).

Immunofluorescence techniques (IFT) are recognized as “gold standard” for the detection of IgG and IgM to VCA, in indirect format (indirect immunofluorescence, IIF), and for EBNA antibodies, by means of the anticomplement immunofluorescence (ACIF) format (7). Nevertheless, IFT are laborious, since they are not automated, require separate assays for each analyte, and

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the interpretation of results is subjective. The use of various serological markers complicates the diagnosis; considering the use of assays detecting IgG and IgM antibodies to VCA, heterophile antibodies, IgG EBV early antigen diffuse (EA-D), and antibodies to EBNA, up to 32 possible serological patterns could be generated (8). Recently, immunoblot (IB) techniques using different EBV antigens have been developed for detecting both IgM and IgG, making possible to obtain EBV seroprofiles in a single determination. The aim of this study was to evaluate an IB assay designed to simultaneously detect IgG antibodies against VCA, EBNA, and EBV EA-D.

MATERIALS AND METHODS

Samples

One hundred and seventeen samples from clinically suspected IM cases were included in the study. The samples were classified as follows:

1. EBV recent infection (RI, 70 samples), if they showed VCA IgM and IgG positive, EBNA antibodies negative (RI 1, 58 samples), positive result to the three markers (RI 2, five samples), or single positive result to VCA IgG (RI 3, seven samples).
2. EBV past infection (PI), if they showed positive result to VCA IgG and EBNA antibodies, with negative result to VCA IgM (29 cases).
3. EBV negative (NEG), if all the markers were negative (18 samples).

Serological Methods

VCA IgM and IgG were studied by IIF, and EBNA antibodies by ACIF, using commercial assays (Merifluor EBV VCA IgM IFA, Merifluor EBV VCA IgG IFA, and Merifluor EBNA test; Meridian Bioscience Inc., Cincinnati, OH). VCA IgM was tested after the IgG was removed from the sample, using an anti-human IgG serum (RF Absorbens, Siemens, Germany).

Method in Evaluation

The kit Euroline Anti-EBV Profile 2 (IgG; Euroimmun, Lübeck, Germany) was evaluated. The assay was designed in an IB format to detect IgG against VCA proteins (gp125 [native, purified by affinity chromatography], VCA p19 [recombinant], and p22 [recombinant]), to EBNA-1 (recombinant), and to EA-D (recombinant). The assay was automatically done in an IB processor (EUROBlotMaster, Euroimmun). Briefly, the strips containing the antigens were soaked in an incubation tray for 15 min at room temperature (RT). After aspiration of the fluid, di-

luted serum samples (1:100) were added and incubated for 30 min at RT. Once the liquid had been aspirated, the strips were washed three times, 5 min each. Then, alkaline phosphatase conjugated anti-human IgG was added, and incubated, the fluid was aspirated, and strips were washed as previously. Finally, substrate solution was added and incubated for 10 min at RT, after which the reaction was stopped by washing the strips three times for 1 min with deionized water. The strips were allowed to dry, and evaluated using the EUROLIneScan software (Euroimmun). Results were expressed as percent probability of primary EBV infection (PPPEBVI): values 70–100% indicated a high probability of primary infection and values <30% excluded it. With values between 30 and 69%, no statement could be made.

To calculate the sensitivity and the specificity of the method in evaluation, indeterminate results were considered the most adverse; that is, the samples were classified as positive if a negative result was expected, and conversely, as negative if a positive one was expected.

When discrepant results were obtained, and depending on the availability of the samples, additional determinations were done: VCA IgG avidity in seropositive samples, as described (5), and EBV PCR in seronegative ones, as described (9).

To calculate the best cut-off points of PPPEBVI, the values were determined by receiver operating characteristic (ROC) curve analysis. The study of qualitative variables was carried out by proportion comparisons (using χ^2 , χ^2 with Yates correction, or χ^2 two-tailed Fisher's exact test when required). To calculate the agreement of the determinations as done by IFT and IB for the different EBV markers, Kappa statistics was used. For the interpretation of results, the following kappa values were considered: <0, less than chance agreement; 0.01–0.20, slight agreement; 0.21–0.40, fair agreement; 0.41–0.60 moderate agreement; 0.61–0.80 substantial agreement; and 0.81–0.99, almost perfect agreement (10). The best PPPEBVI value was defined by ROC curve analysis.

RESULTS

Table 1 shows the results obtained when the samples were tested in the IB assay. All the samples from cases with a typical pattern of primary recent EBV infection (positive result for VCA IgM and IgG, and negative for EBNA antibodies) showed a PPPEBVI higher than 80%. With respect to the five samples showing positive result in the three markers, only one showed a percentage value indicating RI. All the samples with single positive VCA IgG response showed a result indicating RI, six of them had values higher than 80%. Thus, the relative sensitivity of the assay in its ability to diagnose primary EBV infection was 94.3% (66/70).

TABLE 1. General Results Obtained in IB in the Different Groups of Samples

Group	EBV seroprofile			N =	IB ^a			
	VCA-IgM	VCA-IgG	EBNA-Ab		>70	30–69	<30	NV
RI-1	Pos	Pos	Neg	58	58	0	0	0
RI-2	Pos	Pos	Pos	5	1	2	2	0
RI-3	Neg	Pos	Neg	7	7	0	0	0
PI	Neg	Pos	Pos	29	4	5	20	0
Neg	Neg	Neg	Neg	18	4	0	0	14

^aResults are expressed as PPPVEBVI; NV: not valuable.

TABLE 2. Comparison of IFT and IB for the Individual EBV Markers

IB		Reference ^a		Kappa value
		Positive	Negative	
Classification of cases	Recent	66	13	0.688
	No recent	4	34	
p19	Positive	87	4	0.590
	Negative	11	15	
gp125	Positive	90	4	0.652
	Negative	8	15	
VCA ^b	Positive	98	5	0.824
	Negative	0	14	
EBNA	Positive	32	2	0.576
	Negative	2	61	

^aFor the classification of cases, in the reference criteria, positive means recently infected, and negative includes past infected and noninfected. The comparisons of p19 and gp125 were done against VCA by IIF, and the comparison of EBNA was referred to ACIF.

^bConsidering reactivity at least to one antigen (p19 or gp125).

On the other hand, when samples from PI were assayed, four, five, and twenty showed IB result indicating recent, indeterminate, or no recent EBV infections, respectively. Finally, when samples with no prior EBV infection were studied, four showed a result of RI, being the remaining negative for all the antigens included in the IB. Thus, the relative specificity of the assay was 72.3% (34/47). The concordance between IB and the reference was 85.5 (100/117).

Table 2 shows the comparison of IB and IFT for the individual antigens, and the corresponding kappa values. For the classification of cases, the value was 0.688 (substantial agreement); when cases from only RI 1 were considered, the value was 1.0 (perfect agreement, not shown in the table). For p19, gp125, and EBNA, the corresponding values were 0.590, 0.652, and 0.576, respectively (moderate agreement for the three parameters).

Figure 1 shows the best cut-off point for PPPVEBVI: a value of 89% gave the best figures of sensitivity (88.6%)

and specificity (91.5%), with an agreement of 89.7% showing a kappa index for the classification of the cases as RI/not RI of 0.790 (substantial agreement).

Finally, Table 3 lists the samples with discrepant results. Samples 1, 3, and 4, from RI with positive result to EBNA antibodies (RI 2), showed low-avidity IgG, being confirmed the recent primary EBV infection. On the other hand, samples 5 to 8 from PI showed low-avidity IgG as well, confirming as true the classification as RI obtained in the IB assay.

Some of these samples have been part of a previous study done to compare four different systems (two chemiluminescent immunoassays, one immunofiltration, and one ELISA) to establish EBV seroprofiles (11). Sample 2 showed positive result to the three markers in three of four assays; samples 5, 6, and 10 showed PI seroprofile in all the four assays; and sample 9 showed in three of four assays (data not shown).

Samples 14 to 17 were negative according to the reference criterion. They showed a very high PPPVEBVI, with a very low intensity, or absence of response, to the majority of antigens included in the IB. In order to confirm an RI in absence of specific EBV antibody response as assayed by IFT, the samples were tested for the presence of viral genomes; positive result was obtained in sample 16, confirming that this case was due to EBV.

DISCUSSION

Until now IFT are recognized as gold standard assays for EBV serodiagnosis (7). However, these techniques are subjected to some drawbacks, such as the subjective interpretation of results and the difficulties in automation. For this, multiplexed assays such as IB techniques have been suggested as an alternative reference for EBV serology (12). The application of IB assays in an adequate platform to automatize the procedure associated with the appropriate software to interpret the results could be a useful alternative as reference method. This study presents the results of the evaluation of Euroline Anti-EBV Profile 2 (IgG) as done in EUROBlotMaster, with the interpretation of results by EUROLIneScan software. A limitation of the method in evaluation is the cutoff to differentiate primary infections from PIs. With the value established by the manufacturer to confirm recent primary infection (70%), sensitivity and specificity were 94.3 and 72.3%, with a kappa value of 0.688, very similar to the one previously described (0.67) (13). However, if a cut-off value of 89% is considered, the specificity was improved to 91.5% with a moderate decrease in sensitivity to 88.6%.

An important aspect to consider in the design of an IB assay for EBV serology is the selection of antigens to be used. According to our experience, the main aim of this approach should be to substitute IIF for VCA IgG and

Fig. 1. ROC curve. Analysis of sensitivity and specificity to determine the best IB value for diagnosis of EBV infection using as reference IFT assays.

ACIF for EBNA antibodies. For this, a proper selection of antigens must be done. In relation to VCA, although gp125 is recognized as the major antigenic component of VCA complex (14), additional reactivity directed against

other structural proteins and detected by IIF cannot be completely excluded. Among samples from EBV RI, five showed positive gp125 result, being negative for p19, and conversely, two samples were negative for gp125,

TABLE 3. Samples Showing Discrepant Results in the IB and Reference

	IB ^a							
Sample	EAD	p22	EBNA	p19	gp125	PPPVEBVI	Classification	Additional results ^b
RI 2 (positive result for the three EBV markers)								
1	2+	2+	2+	3+	3+	67	Indeterminate	Low-avidity IgG: 16
2	0	0	3+	3+	3+	46	Indeterminate	ND
3	0	3+	2+	3+	3+	14	PI	Low-avidity IgG: 4
4	3+	3+	3+	3+	3+	17	PI	Low-avidity IgG: 4
PI (positive result for the VCA IgG and EBNA antibodies)								
5	0	1(+)	0	3+	2+	87	RI	Low-avidity IgG: 4
6	0	2+	1+	3+	3+	73	RI	Low avidity IgG: 4
7	3+	3+	3+	3+	3+	71	RI	Low-avidity IgG: 4
8	0	1+	2+	3+	1(+)	82	RI	Low-avidity IgG: 4
9	1+	3+	1+	3+	3+	41	Indeterminate	ND
10	3+	3+	3+	3+	3+	58	Indeterminate	ND
11	1+	2+	2+	3+	3+	45	Indeterminate	ND
12	3+	3+	3+	3+	3+	57	Indeterminate	ND
13	3+	3+	3+	3+	3+	35	Indeterminate	ND
NEG (negative results for the three EBV markers)								
14	1+	1(+)	0	1+	1(+)	99	RI	PCR EBV: negative
15	0	2+	0	0	0	97	RI	PCR EBV: negative
16	1(+)	1+	0	0	1(+)	99	RI	PCR-EBV: POSITIVE
17	1+	1(+)	0	1(+)	1(+)	99	RI	PCR EBV: negative

^aFor individual antigens, the results are presented according to the intensity of the corresponding bands; EAD: early antigen diffuse. ^bND: not done.

showing a positive result for p19 (data not shown), thus confirming the usefulness of both proteins in the IB assay. Accordingly, the use of both proteins seems to improve the sensitivity of VCA detection, since the kappa value was increased to 0.824 (almost perfect agreement) when VCA IgG by IIF is compared with the presence of IgG against p19 and/or gp125 in IB (Table 2).

On the other hand, EBV p22 is a viral late complex associated with virion particles (15). For this, IgG against p22, as happens with EBNA antibodies, should be formed in the late phase of infection, as stated in the insert of the reagent. However, contrary to expectations, the comparison of results of IgG p22 against EBNA by ACIF provides a low kappa value (0.312, fair agreement; data not shown).

Multiplexed assays such as IB techniques could be considered as alternative approaches to the conventional IFT for the establishment of EBV seroprofiles. These approaches have a lower cost, reduce hands-on time, and minimize the risk derived from the aliquoting of samples. However, efforts to better define the cut-off value and select the adequate antigens included in the test should be made in order to improve the performance of the assay in evaluation.

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