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# Evaluation of an Immunofiltration Assay That Detects Immunoglobulin M Antibodies against the ZEBRA Protein for the Diagnosis of Epstein-Barr Virus Infectious Mononucleosis in Immunocompetent Patients▽

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**The performance of an immunofiltration assay (IMFA) that detects immunoglobulin M (IgM) antibodies to the Epstein-Barr virus (EBV) ZEBRA (BamHI Z EBV replication activator) protein was evaluated for the diagnosis of EBV infectious mononucleosis (IM) in immunocompetent patients. The test panel consisted of 47 sera displaying an EBV-specific antibody profile compatible with an acute primary EBV infection from patients with clinical and biological features of EBV IM, 20 sera from healthy individuals either with a past EBV infection or who were EBV seronegative, 20 sera displaying an equivocal EBV antibody pattern (viral capsid antigen IgG positive [VCA IgG$^+$], VCA IgM$^+$, and EBV nuclear antigen-1 IgG$^+$), and 15 sera obtained from patients with a mononucleosis-like syndrome owing to cytomegalovirus, human herpesvirus 6, or parvovirus B19. Overall, the sensitivity and the specificity of the assay were found to be 92.5%, and 97.3%, respectively. The sensitivity of the assay for the diagnosis of heterophile antibody-negative EBV IM was 86.2%. The IMFA is rapid, easy to perform, and, thus, suitable for point-of-care testing, and it may be used as a first-line test for the diagnosis of acute EBV IM in immunocompetent patients.**

Diagnosis of Epstein-Barr virus (EBV) infectious mononucleosis (IM) is commonly made on the basis of characteristic clinical manifestations and the detection of heterophile antibodies (HA). Nevertheless, HA may be absent, particularly in young children (14) but also in as many as 20% of adults with EBV IM (7). In these cases, demonstration of the presence of EBV viral capsid antigen (VCA) immunoglobulin G (IgG) and/or IgM antibodies, along with the absence of IgG antibodies to EBV nuclear antigen-1 (EBNA-1), allows the diagnosis of EBV primary infection (9). Detection of EBV-specific antibodies is accomplished by the use of commercial enzyme immunoassays, indirect immunofluorescence assays, line blot immunoassays (9), or, as established more recently, a multiplexed bead assay (3). These methods have long turnaround times, are labor-intensive, or require specific instruments or skilled technologists for their performance. In addition, interpretation of EBV VCA IgG/IgM and EBNA-1 IgG reactivity profiles is not always straightforward (9).

The ZEBRA (BamHI Z EBV replication activator) protein is encoded by the immediate early BZLF1 gene. ZEBRA is expressed during the lytic cycle in EBV-permissive cells and plays a critical role in transactivating several immediate early, early, and late EBV genes (5). Antibodies against ZEBRA are produced during primary EBV infection (11, 15, 18), and thus, the detection of ZEBRA-specific IgMs may allow an early diagnosis of EBV IM. In the present study, we evaluated a rapid and easy-to-perform immunofiltration assay (IMFA) detecting IgMs to the EBV ZEBRA protein for the biological diagnosis of IM in immunocompetent patients.

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## MATERIALS AND METHODS

**Serum specimens.** A total of 102 sera submitted to our laboratory from 2005 to 2008 for routine EBV-specific antibody testing for diagnostic purposes were evaluated in this study. Serum samples were stored at −20°C immediately after separation and were retrieved for further analysis.

**Immunoassays.** VCA IgG, VCA IgM, and EBNA-1 IgG antibodies were detected by enzyme immunoassays (EIAs) (Captia) from Trinity-Biotech (Bray, Ireland). VCA IgG avidity studies were performed by following a previously published protocol (6, 8, 12). In brief, VCA IgG avidity was determined with the VCA IgG EIA (Captia), with the first wash step modified to include two washes (five minutes each) with a washing buffer containing urea (8 M). The avidity index value (as a percentage) was calculated as follows: (absorbance of VCA IgGs in the presence of urea/absorbance of VCA IgG in the absence of urea) × 100. IgG avidity index values less than 50% were considered compatible with a recent primary EBV infection (8). In our experience, sera from patients with a remote EBV infection (VCA IgG positive [VCA IgG$^+$]/EBNA-1 IgG$^+$/VCA IgM$^-$/HA-negative) give VCA IgG avidity values greater than 75%, whereas those for sera from patients with demonstrated recent primary EBV infection (VCA IgM$^+$/VCA IgG$^+$/EBNA-1 IgG$^+$/HA-positive) are less than 50% (data not shown). HA were detected by a differential agglutination assay (I.M. kit; Microgen, Surrey, Great Britain). Parvovirus B19-specific IgG and IgMs were detected by an EIA from Biotrin International (Dublin, Ireland). Cytomegalovirus (CMV)-specific IgG and IgM antibodies were detected by the respective Architect or Axsym CMV assay (Abbott Diagnostics, IL). IgG and IgM antibodies to human herpesvirus-6 (HHV-6) were detected by an EIA from Panbio (Queensland, Australia). For all EIAs, interpretation of the results was made according to the instructions of the respective manufacturer.

**IMFA.** The IMFA (flowthrough device) is based on an assay principle in which the sample and a variety of reagent and wash solutions are sequentially absorbed through a porous membrane containing spots or lines of immobilized analyte-specific or control antibodies (reviewed in reference 17). The Immunoquick Filtration EBV M assay (referred to as the IMFA herein) was performed by following the instructions of the manufacturer (BioSynex Immunodiagnostic, Strasbourg Cedex, France). Briefly, a volume of 25 μl of serum was diluted in 1.5 ml of dilution buffer in a plastic tube. The diluted sample was then poured directly from the tube into the device window and allowed to drain for 15 s. The conjugate (1.5 ml) was then added and allowed to drain through the device window (15 s). The washing buffer (1.5 ml) was added next, and the reaction was read as soon as the buffer was fully drained through the device window. The appearance of test 1 (T1) (ZEBRA synthetic peptide) and control (immobilized protein A) blue bands indicated the presence of ZEBRA-specific IgM antibodies. The appearance of the control band in the absence of the T1 band indicated

TABLE 1. Performance of the IMFA with sera displaying different EBV-specific antibody profiles as determined by EIA

| Serum group (no. of samples) | EBV-specific antibody profile | No. (%) of samples with indicated IMFA result | | |
|---|---|---|---|---|
| | | Positive | Weakly positive | Negative |
| A (24) | VCA $IgG^-/IgM^+$, EBNA-1 $IgG^-$ | 17 (70) | 4 (16) | 3 (14) |
| B (23) | VCA $IgG^+/IgM^+$, EBNA-1 $IgG^-$ | 17 (73) | 4 (17) | 2 (10) |
| C (10) | VCA $IgG^-/IgM^-$, EBNA-1 $IgG^-$ | 0 (0) | 0 (0) | 10 (100) |
| D (10) | VCA $IgG^+/IgM^-$, EBNA-1 $IgG^+$ | 0 (0) | 0 (0) | 10 (100) |
| E (20) | VCA $IgG^+/IgM^+$, EBNA-1 $IgG^+$ | 2 (10) | 11 (55) | 7 (35) |
| F[a] (15) | | 1 (6) | 1 (6) | 13 (88) |

[a] Sera from patients with a mononucleosis-like syndrome owing to CMV, parvovirus B19, or HHV-6. Ten sera lacked EBV-specific antibodies, four sera were VCA $IgG^+/IgM^-$ and EBNA-1 $IgG^+$, and one serum sample was VCA $IgG^+/IgM^-$ and EBNA-1 $IgG^+$.

a negative result, while the absence of both bands invalidated the test. The results of the tests were interpreted independently by three observers. The appearance of a faint T1 band was categorized as a weakly positive result.

**Detection of EBV DNA in serum.** A commercial herpesvirus multiplex real-time PCR assay (RealCycler HHHH-0; Progenie Molecular, Valencia, Spain), carried out with the SmartCycler instrument (Cepheid, Sunnyvale, CA), was used for the detection of EBV DNA in sera (200 μl).

**Test panel.** The criteria used to define EBV serostatus (susceptible, acutely infected, and having a past infection) were based on consensus EBV-specific antibody profiles (9). The test panel included the following categories of serum specimens. Group A ($n$ = 24) consisted of sera obtained from patients with acute (early) primary EBV infection (VCA $IgG^-$, VCA $IgM^+$, EBNA-1 $IgG^-$, follow-up serum specimens showing VCA IgG seroconversion, clinical manifestations compatible with EBV IM, and absence of IgM antibodies to CMV, HHV-6, and parvovirus B19). Eleven of the 24 sera tested positive in the HA assay; the median age of patients (15 males and 9 females) belonging to this group was 4 years (range, 1 to 16 years). Group B ($n$ = 23) consisted of sera drawn from patients (12 males and 11 females) with an acute primary EBV infection (VCA $IgG^+$, VCA $IgM^+$, EBNA-1 $IgG^-$, VCA IgG avidity index values of <50%, clinical manifestations compatible with IM, and absence of IgM antibodies to CMV, HHV-6, and parvovirus B19). Twelve of these sera tested negative in the HA assay. The median age of patients in this group was 5 years (range, 3 to 25 years). Group C ($n$ = 10) consisted of sera drawn from EBV-susceptible (VCA $IgG^-$, VCA $IgM^-$, and EBNA-1 $IgG^-$) healthy individuals (six females and four males); the median age for this group was 15 years (range, 10 to 65 years). Group D ($n$ = 10) consisted of sera obtained from healthy individuals (six males and four females) with a past EBV infection (VCA $IgG^+$, VCA $IgM^-$, EBNA-1 $IgG^+$, VCA IgG avidity index values of >80%, and negative for HA). The median age of patients in this group was 6 years (range, 3 to 8 years). Group E ($n$ = 20) consisted of sera drawn from patients (10 females and 10 males) displaying an equivocal EBV-specific antibody profile (VCA $IgG^+$, VCA $IgM^+$, and EBNA-1 $IgG^+$) compatible with either late primary EBV infection or subclinical EBV reactivation. Three of these sera tested positive in the HA assay. The median age of patients in this group was 3 years (range, 2 to 14 years). None of these sera tested positive in the CMV-, HHV-6-, and parvovirus B19-specific IgM assays. Group F ($n$ = 15) consisted of sera obtained from patients (10 females and 5 males) with a mononucleosis-like syndrome owing to CMV ($n$ = 5), parvovirus B19 ($n$ = 5), or HHV-6 ($n$ = 5). Ten of these sera were seronegative for EBV, four sera displayed an EBV serological profile compatible with a past EBV infection, and one serum sample was VCA $IgG^+$, VCA $IgM^-$, and EBNA-1 $IgG^-$. None of these sera tested positive in the HA assay. The median age of patients in this group was 8 years (range, 1 to 15 years).

## RESULTS

**Performance of the IMFA with sera from patients with acute EBV IM.** Twenty-one sera from patients in the early phase of acute IM (group A) tested positive (four sera gave a weak positive result) in the IMFA (Table 1). The remaining three sera tested negative in the IMFA, the EBV DNA assay, and the HA test. Twenty-one sera from patients with acute EBV IM (group B) tested positive in the IMFA. The other two sera tested negative in the IMFA and in the EBV DNA assay and displayed VCA IgG avidity index values around 50%. One of the two sera was found to be positive in the HA test. The diagnostic efficacy of the IMFA for sera lacking heterophile antibodies was assessed. Twenty-five of the 47 sera in groups A and B tested negative in the HA assay (Table 2). IgMs to ZEBRA were detected by the IMFA in 21 of the 25 sera. The four nonreactive samples tested negative in the EBV DNA assay. One of these serum specimens tested positive for VCA IgGs and had a VCA IgG avidity index value of 48%. All of the 22 sera testing positive in the HA test gave a positive result in the IMFA.

TABLE 2. Performance of the IMFA with sera from patients with EBV IM testing either positive or negative in the HA agglutination assay

| EBV-specific antibody profile | Result of HA test (no. of sera) | No. of samples with indicated IMFA result | | |
|---|---|---|---|---|
| | | Positive | W+[a] | Negative |
| VCA $IgG^-$/VCA $IgM^+$/EBNA-1 $IgG^-$ | HA positive (11) | 9 | 2 | 0 |
| | HA negative (13) | 7 | 3 | 3 |
| VCA $IgG^+$/VCA $IgM^+$/EBNA-1 $IgG^-$ | HA positive (11) | 10 | 1 | 0 |
| | HA negative (12) | 8 | 3 | 1 |

[a] W+, weakly reactive.

**Performance of the IMFA with sera from susceptible and non-acutely EBV-infected individuals.** None of the sera obtained from either EBV-seronegative individuals (group C) or individuals with a past EBV infection (group D) yielded a positive reaction in the IMFA.

**Performance of the IMFA with sera displaying an equivocal EBV-specific antibody profile.** The performance of the IMFA was also evaluated in sera ($n$ = 20) displaying an equivocal EBV-specific antibody pattern (VCA $IgG^+$, VCA $IgM^+$, and EBNA-1 $IgG^+$) (group E). This serological profile is frequently detected in routine EBV-specific antibody testing and may be observed either in the late phase of acute IM or in the setting of EBV reactivation. Thirteen of the 20 sera tested positive (11 reacted weakly) in the IMFA (Table 1). As shown in Table 3, 6 of the 13 sera positive by the IMFA had VCA IgG avidity index values greater than 50% and tested negative in both the EBV DNA assay and the HA test. These sera gave a weak positive signal in the IMFA. The remaining seven sera positive by the IMFA had VCA IgG avidity index values less than 50% (determination of VCA IgG avidity could not be performed in two serum specimens). Four and three of the 7 sera tested positive in the EBV DNA assay and in the HA test, respectively. Seven of the 20 sera tested negative in the IMFA. All of these sera had VCA IgG avidity index values greater than 50% and tested negative in both the EBV DNA assay and the HA test.

**Performance of the IMFA with sera from patients with a mononucleosis-like syndrome caused by other viruses.** Fifteen sera from patients with primary symptomatic infections owing to CMV, HHV-6, or parvovirus B19 (group F) were tested in the IMFA. Two out of the 15 sera were found to be reactive. One of these two sera displayed an EBV-specific antibody pattern compatible with a past EBV infection and tested negative in the EBV DNA test. The other serum specimen had a

TABLE 3. Performance of the IMFA with sera displaying an equivocal EBV-specific antibody profile (VCA IgG$^+$, VCA IgM$^+$, EBNA-1 IgG$^+$)

| Serum no. | Presence of HA | IMFA result | VCA IgG avidity (%) | Presence of EBV DNA in serum |
|---|---|---|---|---|
| 1 | − | − | 96 | − |
| 2 | − | − | 75 | − |
| 3 | − | − | 100 | − |
| 4 | − | − | 81.5 | − |
| 5 | − | − | 75 | − |
| 6 | − | W+[a] | 7 | − |
| 7 | − | W+ | 91.6 | − |
| 8 | − | − | 100 | − |
| 9 | − | − | 100 | − |
| 10 | − | W+ | 33 | − |
| 11 | − | W+ | 68 | − |
| 12 | − | W+ | 87.7 | − |
| 13 | − | W+ | 97.3 | − |
| 14 | − | W+ | 82 | − |
| 15 | − | W+ | 56.5 | − |
| 16 | + | + | 17.8 | + |
| 17 | + | + | 39.3 | + |
| 18 | + | W+ | 12.39 | + |
| 19 | − | W+ | ND[b] | + |
| 20 | − | W+ | ND | + |

[a] W+, weakly positive.
[b] ND, not done.

serological profile compatible with a primary EBV infection (VCA IgG$^+$, VCA IgM$^-$, and EBNA-1 IgG$^-$) and also tested negative in the EBV DNA assay. No sufficient volume of these sera was available for VCA IgG avidity analysis.

**Sensitivity and specificity of the IMFA.** For the calculation of the sensitivity and specificity of the IMFA, the following criteria were adopted: (i) the sera included in groups A and B ($n = 47$) and those in group E that yielded low VCA IgG avidity index values and/or tested positive in the EBV DNA assay and that belonged to patients with clinical features of acute IM ($n = 7$) were considered true positives for the diagnosis of EBV IM and (ii) the sera in groups C and D ($n = 20$), which displayed EBV-specific antibody profiles compatible with past EBV infection or no EBV infection, and 14 of the 15 sera from patients with primary infection owing to other viruses (from group F; the serum specimen displaying the VCA IgG$^+$, VCA IgM$^-$, and EBNA-1 IgG$^-$ EBV-specific antibody profile was excluded because of the possibility of an EBV and HHV-6 coinfection) were considered true negatives for the diagnosis of EBV IM. On the basis of the criteria mentioned above, the IMFA had a sensitivity of 92.5% (86.2% if only sera testing negative in the HA assay were considered for analysis) and a specificity of 97.3%.

## DISCUSSION

The availability of a rapid, easy-to-perform, sensitive, and specific antibody assay for the diagnosis of EBV IM would represent a great improvement in EBV serology. In the present study, we evaluated a rapid and simple IMFA for the diagnosis of EBV IM in immunocompetent patients by using a well-characterized panel of sera. This test attempts to detect IgMs to EBV ZEBRA, a key protein for the initiation of the lytic cycle in EBV-permissive cells (5). IMFAs have been previously shown to perform optimally for the diagnosis of a number of infectious diseases, such as congenital toxoplasmosis and syphilis (10, 19).

To our knowledge, there are no published data in relation to the clinical value of assessing the presence of IgMs to ZEBRA for the diagnosis of EBV IM except for a study evaluating the performance of a capture enzyme-linked immunosorbent assay that detects antibodies to a recombinant ZEBRA protein (18). The ZEBRA immunofiltration test was found to be highly sensitive (92.5%) and specific (97.3%) for the diagnosis of EBV IM. In effect, 42 of 47 sera (89.3%) displaying a antibody profile typical of an acute primary EBV infection (VCA IgG$^+$ or VCA IgG$^-$, VCA IgM$^+$, and EBNA-1 IgG$^-$) gave a positive result in the IMFA. This figure is in contrast with that (14%) previously reported by another group (18). The most likely explanation for this discrepancy is the different natures of both the antigens and the immunoassay formats employed (a prokaryotic recombinant ZEBRA protein and a sandwich capture enzyme-linked immunosorbent assay were used in the above-mentioned study, whereas a combination of immunogenic ZEBRA-derived synthetic peptides were employed in the evaluated IMFA in this study). Five of the 47 sera tested negative in the IMFA. These sera also tested negative in the EBV DNA PCR assay, which has been shown to be frequently positive within the first 3 weeks after the onset of disease (2, 4). In addition, the two sera in which VCA IgGs were detected displayed rather high VCA IgG avidity values (around 50%), indicating that these samples may have been drawn relatively late after the onset of infection, once IgMs to ZEBRA had already been cleared.

A remarkable feature of the IMFA was its performance with sera testing negative in the HA assay. The sensitivity of the IMFA in HA-negative sera was 86.2%, which makes it particularly valuable for the diagnosis of EBV IM in young children.

The IMFA was found to be highly specific, not only when sera from either EBV-seronegative individuals or individuals with past EBV infection were considered for analysis (none tested positive) but also when sera from patients either remotely infected by EBV or not infected with EBV and displaying a mononucleosis-like syndrome owing to CMV, HHV-6, or parvovirus B19—in which reappearance of VCA IgMs is frequently observed (1, 12, 16)—were evaluated. Two serum specimens in the last group gave a positive result in the IMFA. One of these sera, however, displayed an EBV-specific antibody pattern compatible with a primary EBV infection (VCA IgG$^+$, VCA IgM$^-$, EBNA-1 IgG$^-$), so that an EBV and HHV-6 coinfection could not be ruled out. The other sera belonged to a patient with a past EBV infection who developed a primary symptomatic infection caused by HHV-6. A subclinical EBV reactivation concurrent with primary infection by HHV-6 may well explain the reappearance of serum IgMs to ZEBRA in this patient.

The performance of the IMFA was also assessed with sera displaying an equivocal EBV-specific antibody pattern (VCA IgG$^+$, VCA IgM$^+$, and EBNA-1 IgG$^+$). This EBV-specific antibody pattern usually occurs in late primary EBV infection or during subclinical EBV reactivation. Nevertheless, it may also be observed, albeit at a low frequency, relatively early after the onset of IM clinical manifestations (13) or in the setting of a CMV, HHV-6, or parvovirus B19 primary infection, in which

reappearance of VCA IgMs is not uncommon (1, 12, 16). Discrimination between these possibilities is often difficult, even if results of ancillary tests (VCA IgG avidity analysis and serum EBV DNA PCR) are available, since kinetics of IgG avidity maturation may vary widely between individuals (6, 8), and EBV DNA is only transiently detected in serum during EBV IM (2, 4). Thirteen of these sera belonged to patients presumably in the late stage of a primary EBV infection, as inferred from the review of clinical charts and the demonstration of high VCA IgG avidity index values in the absence of EBV DNA in sera. The IMFA gave a weak positive result for 6 of these 13 sera, indicating that in certain cases, IgMs against ZEBRA may persist at low levels during IM convalescence. Seven of the 20 sera belonged to patients in the acute phase of EBV IM. These sera had low VCA IgG avidity index values (<50%) and/or detectable EBV DNA. The seven sera tested positive in the IMFA.

In conclusion, our data indicate that the IMFA is a sensitive and specific test for the diagnosis of acute EBV IM. In this sense, it was found to be particularly useful for the diagnosis of EBV IM in children not developing HA.

The IMFA is rapid, easy to perform and, thus, suitable for point-of-care testing, and it may be used as a first-line assay for the diagnosis of EBV IM, especially in young children. Further studies are required, however, to determine to what extent this test may replace conventional EBV-specific antibody assays for the diagnosis of acute EBV IM.

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