Comparison of Three Automated Immunoassay Methods for the Determination of Epstein-Barr Virus-Specific Immunoglobulin M

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In this study we compared the performances of three commercially available Epstein-Barr virus (EBV) immunoglobulin M (IgM) assays on highly automated immunoassay platforms: BioPlex 2200 (Bio-Rad Laboratories), Immulite 2000 (Siemens Healthcare Diagnostics), and Liaison (DiaSorin). As a confirmatory method, immunoblotting was performed. The specificity of the three EBV IgM assays was evaluated by testing 293 selected sera from patients with various infectious and noninfectious diseases. After the exclusion of 30 samples, the specificities were 96.2% for Liaison, 98.1% for Immulite, and 97.0% for BioPlex. For evaluation of the sensitivity, samples from 70 consecutive patients with a positive heterophile antibody test were examined, irrespective of clinical or biological findings. After the exclusion of six samples, the sensitivities were 89.1% for Liaison, 84.4% for Immulite, and 89.1% for BioPlex. Finally, in a prospective study performed with 500 samples obtained from consecutive patients and sent in by general practitioners, we also determined Epstein-Barr nuclear antigen IgG and viral capsid antigen IgG in a two-phase approach. Concordance of the EBV serologic status was 96.2% between Liaison and Immulite, 96.4% between Immulite and BioPlex, and 97.8% between BioPlex and Liaison. The three EBV IgM immunoassays that we evaluated have acceptable and comparable performances.

**MATERIALS AND METHODS**

**Patients and samples.** To evaluate the sensitivity of the three EBV IgM methods, we collected serum samples from 70 consecutive patients with a positive heterophile antibody test (Clearview IM; Unipath, Bedford, United Kingdom) without taking any clinical or biological findings into consideration. Only samples sent in by general practitioners were used. This approach provides a patient population with a high probability of acute EBV infections without favoring any of the three methods under evaluation. If different results were obtained between the three EBV IgM tests or if a negative result was obtained in all three methods, an EBV IgM immunoblot analysis was performed as a confirmatory method.

For the second part of our study, evaluating the specificity of the three EBV IgM assays, we selected 293 sera from four patient groups. The first group consisted of sera from 126 patients with disturbed immune regulation: antinuclear antibodies (n = 21), antithyroid-peroxidase antibodies (n = 27), rheumatoid factor (n = 39), antiecodoitin IgM antibodies (n = 5), intrinsic factor antibodies (n = 6), chronic lymphocytic leukemia (n = 16), and IgM paraproteinemia (n = 12). The second group consisted of 117 patients with various acute and chronic infectious diseases: acute hepatitis A (n = 15), chronic hepatitis B (hepatitis B surface antigen positive, n = 26), chronic hepatitis C (hepatitis C RNA positive, n = 11), human immunodeficiency virus (HIV RNA positive, n = 12), acute toxoplasmosis as shown by an IgG seroconversion (n = 9), and acute herpes simplex virus infection as shown by a herpes simplex IgG seroconversion (n = 4). In addition, in this second group 18 sera were included that contained immunoblot-confirmed IgM antibodies against Borrelia burgdorferi sensu lato and 22 sera from patients with evidence of a Treponema pallidum infection (Rapid Plasma Reagin and Treponema pallidum hemagglutination assay positive). The third group consisted of 30 patients with acute parvovirus B19 infections (n = 15) and acute rubella virus infections (n = 15). This third group was considered different from the second group because we have previously shown that during the acute phase of these infections, false-positive EBV IgM results can occur on Liaison (2). The fourth group consisted of 20 patients with an acute CMV infection. In this group the diagnosis of an acute CMV infection was ascertained by either a CMV IgG seroconversion (n = 8) or a significant CMV IgG titer change (i.e., >4, n = 12), without concurrent changes in the EBV VCA IgG titers. Moreover, EBNA IgG was positive in these 20 patients, virtually excluding an acute EBV infection. In these 293 sera, if a positive EBV IgM result was obtained in any of the three methods, an EBV IgM immunoblot was performed as a confirmatory method.

In the third part of the study, we prospectively evaluated the serologic EBV status on samples from 500 consecutive patients (335 females and 165 males;

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mean age, 29 years), which were sent in by general practitioners with a request for EBV IgM determination. On these samples we determined, in addition to the EBV IgM, the EBV IgG antibodies in a two-phase approach: Epstein-Barr nuclear antigen (EBNA) IgG was assessed and, if the sample was found to be negative, viral capsid antigen (VCA) IgG was assessed. The purpose here was to evaluate the effect of possible erroneous EBV IgM results on the final serologic EBV status, keeping in mind that EBNA IgG virtually excludes a recent EBV infection, and therefore its presence in serum has a major impact on the final serological interpretation. In this patient group, if a different serological interpretation between the three methods due to discrepancies in the EBV IgM results was obtained, EBV IgM immunoblotting was performed as a confirmatory method.

During the evaluation of the results from the present study, we suspected possible false-negative EBV VCA IgG results from the BioPlex analysis. Although it was not our primary intention to evaluate the EBV VCA IgG methods, we considered these results important, and we decided to further evaluate this possible problem. We therefore analyzed 56 samples from our serum repository: paired serum samples from 15 patients with an acute EBV infection, as shown by a VCA IgG seroconversion (\( n = 13 \)) or significant titer rise (i.e., \( >3, n = 2 \)). From eleven patients three or more samples were available. In this fourth and added part of our study an important selection bias was present since these samples were all selected on results obtained from Liaison. In this fourth part we also further investigated the sera from the first part of the study (the heterophile antibody-positive group) by determining VCA IgG on the three platforms.

Methods. EBV VCA IgM, VCA IgG, and EBNA IgG determinations were performed on three automated immunoassay platforms according to the manufacturer’s instructions: BioPlex 2200 (Bio-Rad Laboratories, Hercules, CA), Immulite 2000 (Siemens Healthcare Diagnostics, Los Angeles, CA), and Liaison (DiaSorin, Saluggia, Italy). The applied cutoffs for the Liaison assays were 40 mU/liter for VCA IgM, 20 mU/liter for VCA IgG, and 20 mU/liter for EBNA IgG. For the assays performed using BioPlex and Immulite a cutoff of 1.1 (ratio) was used.

To resolve possible discrepancies or unexpected results, immunoblotting was performed using the EBV-profile 2 Euroline IgM (Euroimmun, Lübeck, Germany), determining IgM antibodies to the following EBV antigens: VCA gp125, VCA p19, EBNA-1, p22, and EA-D. The immunoblot test strips were automatically incubated by using the EUROBlotMaster system (Euroimmun) and objectively evaluated by using the EUROLineScan (Euroimmun) software program.

Statistical evaluation. For statistical comparison of the three methods, a chi-square test for the comparison of proportions was applied by using Medcalc software (version 9.4; Mariakerke, Belgium). P values of <0.05 were considered significant.

RESULTS

Sensitivity evaluation. In the group of 70 patients with a high probability of an acute EBV infection, six samples were eliminated from the final calculation. Five samples were considered to be EBV IgM negative because all three EBV IgM tests were negative and also immunoblotting gave a negative result. One sample was eliminated because of a borderline result on immunoblotting (weakly positive band for VCA p19). The results for this sample were positive on Liaison (53 mU/liter), positive on BioPlex (2.9 [ratio]), and negative on Immulite (0.9 [ratio]). Thus, the final number of samples used in this population was 56.

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The sensitivities of the three EBV IgM methods in the study population were 89.1% for Liaison, 84.4% for Immulite, and 89.1% for BioPlex. These results were not statistically significantly different.

Specificity evaluation. The results from the group of 293 sera from various immune disorders and infectious diseases are shown in Table 2. Considering the very high number of positive EBV IgM results in the CMV group, not only for the three methods under evaluation but frequently also on immunoblotting, we did not include these 20 samples in the specificity calculation. We also eliminated for the specificity calculation 10 samples with a positive or indeterminate result on immunoblotting. Thus, the specificities calculated based on 263 samples were 96.2% for Liaison, 98.1% for Immulite, and 97.0% for BioPlex. These results were not statistically significantly different. Since an important selection bias was present in this part of the study, with possible negative interpretative effects on Liaison performance, further recalculation of assay specificities excluding the 15 acute rubella samples yielded values of 98.4% for Liaison, 98.4% for Immulite, and 97.2% for BioPlex. These results were also not statistically significantly different.

Prospective study. For the serologic EBV status, the interpretation of the antibody profile was performed according to the rules shown in Table 3. In general, the presence of EBNA IgG antibodies was considered a hallmark for a previous EBV infection, irrespective of the presence of EBV IgM antibodies (11). Table 4 shows the comparison of the final serologic EBV status obtained on the three platforms using these easy interpretative rules. The methods agreed on EBV serologic status in 96.2% of cases between Liaison and Immulite, in 96.4% of cases between Immulite and BioPlex, and in 97.8% of cases between BioPlex and Liaison. These results were not statistically significantly different.

In this prospective part, 11 patients (2.2%) had a possible recent EBV infection according to one of the three methods. The detailed serological results from these 11 patients are shown in Table 5. In only one patient did the three methods agree on a possible recent infection. In the other ten patents a discrepancy was observed between the three methods. One case (no. 1225) was misclassified by Liaison due to a false-positive EBV IgM and/or a false-negative EBNA IgG, one case (no. 1244) was misclassified by both Liaison and BioPlex due to a false-positive EBV IgM, one case (no. 1309) was misclassified by Immulite due to a false-negative EBV IgM, and one case (no. 1408) was misclassified by BioPlex due to a false-negative EBV IgM. In one case (no. 1297) we were not able to exactly determine the serologic EBV status, due to an inconclusive immunoblot (weakly positive band VCA p19). In the remaining five patients we considered the discrepancies in the sero-

### Table 1. EBV VCA IgM results from the 12 samples with unexpected results from the sensitivity evaluation

| Study ID | Liaison | Cutoff<sup>a</sup> | BioPlex |
|----------|---------|-----------------|---------|
| 620      | 100 (+) | 0.5 (–)         | 1.7 (+) |
| 624      | 68 (+)  | 5.9 (+)         | 0.9 (–) |
| 631      | 75 (+)  | 1.4 (+)         | 0.6 (–) |
| 632      | 125 (+) | 0.9 (–)         | 2.8 (+) |
| 642      | 21 (–)  | 0.5 (–)         | 0.2 (–) |
| 654      | 130 (+) | 0.7 (–)         | 2.7 (+) |
| 655      | 12 (–)  | 0.1 (–)         | 0.2 (–) |
| 658      | 13 (–)  | 0.2 (–)         | 0.6 (–) |
| 663      | 25 (–)  | 0.5 (–)         | 1.5 (+) |
| 666      | 10 (–)  | 0.1 (–)         | 0.2 (–) |
| 671      | 22 (–)  | 0.9 (–)         | 1.1 (+) |
| 672      | 10 (–)  | 0.1 (–)         | 0.2 (–) |

<sup>a</sup> Values are expressed as mU/liter for Liaison and as ratios for BioPlex and Immulite. All samples were positive for EBV IgM on immunoblotting. The applied cutoffs for the EBV IgM assays were 40 mU/liter for Liaison and 1.1 (ratio) for BioPlex and Immulite. The interpretation as positive or negative is indicated in parentheses.
logic status only minor, and the obtained data were insufficient to point out the correct method. However, in three of these cases (cases 1187, 1215, and 1309) we observed an interesting phenomenon: only Liaison and Immulite showed strongly positive titers for EBV VCA IgG, whereas the BioPlex result was clearly negative. These three cases, probably in the transient phase of the infection, raised our suspicion of possible false-negative EBV VCA IgG results in BioPlex.

VCA evaluation. In the fourth and added part of our study, we analyzed 15 seroconversion sets from our serum repository, and the following results were obtained. On Immulite, in four cases no seroconversion or significant titer change could be observed, and this was due to strong positivity of VCA IgG in the first sample of a panel. On BioPlex, in six cases no seroconversion or significant titer change could be shown but, in contrast to Immulite, this absence of titer changes was due to persistent VCA IgG negativity of the follow-up samples. In the other nine cases where a seroconversion could be demonstrated on BioPlex, this evolution occurred very slowly: the median time to show a VCA IgG seroconversion or significant titer change on Liaison and on Immulite was 20 days, whereas on BioPlex it was 60 days.

We also analyzed in this added part of the study the VCA IgG on the 64 samples from the heterophile antibody-positive samples. Based on Liaison analysis, eight samples (12.5%) were negative for VCA IgG, whereas the BioPlex analysis, 58 samples (90.6%) were negative based on Immulite analysis, but 58 samples (90.6%) were negative based on BioPlex analysis (statistically significantly different, \( P < 0.001 \)).

**DISCUSSION**

To our knowledge, no data have been published on the performance of the Immulite EBV IgM. As we have shown, this method performs comparably to both BioPlex and Liaison.

| Group (no. of samples) | Liaison EBV IgM (false<sup>a</sup>) | Immulite EBV IgM (false<sup>a</sup>) | BioPlex EBV IgM (false<sup>a</sup>) | Immunoblot IgM<sup>a</sup> or indeterminate |
|------------------------|-----------------------------------|-----------------------------------|-----------------------------------|------------------------------------------|
| Antinuclear antibodies (21) | 0 (0) | 1 (1) | 0 (0) | 0 |
| Antithyroid-peroxidase antibodies (27) | 2 (0) | 3 (1) | 2 (0) | 2 |
| Rheumatoid factor (39) | 1 (0) | 1 (0) | 1 (0) | 1 |
| Anticardiolipin IgM (5) | 1 (0) | 1 (0) | 1 (0) | 1 |
| Anti-intrinsic factor antibodies (6) | 0 (0) | 0 (0) | 1 (1) | 0 |
| Chronic lymphocytic leukemia (16) | 1 (1) | 1 (0) | 1 (0) | 1 |
| Paraproteinemina IgM (12) | 0 (0) | 0 (0) | 0 (0) | 0 |
| Acute hepatitis A (15) | 0 (0) | 2 (1) | 2 (1) | 1 |
| Chronic hepatitis B (26) | 0 (0) | 2 (1) | 2 (1) | 1 |
| Chronic hepatitis C (11) | 0 (0) | 0 (0) | 0 (0) | 0 |
| HIV (12) | 0 (0) | 0 (0) | 0 (0) | 0 |
| Acute toxoplasmosis (9) | 2 (1) | 0 (0) | 3 (2) | 1 |
| Acute herpes simplex virus (4) | 0 (0) | 0 (0) | 0 (0) | 0 |
| *Brucella burgdorferi* IgM (18) | 2 (2) | 1 (1) | 3 (3) | 0 |
| Syphilis (22) | 1 (0) | 1 (0) | 0 (0) | 2 |
| Acute parvovirus B19 (15) | 0 (0) | 0 (0) | 0 (0) | 0 |
| Acute rubella virus (15) | 7 (6) | 2 (1) | 2 (1) | 1 |
| Acute CMV (20) | 11 (2) | 8 (1) | 12 (3) | 10 |

Total (293) 28 (12) 21 (6) 28 (11) 20

<sup>a</sup> The number of false-positive (false<sup>a</sup>) EBV IgM results is shown in parentheses, e.g., “2 (1)” should be interpreted as two positive EBV IgM results, one of which was false positive.
The results from the present study are in agreement with previous reports on the performance of BioPlex and Liaison (4, 7, 14). There are, however, minor differences between the various publications on BioPlex and Liaison, and these are probably not only attributable to sample size in the different studies but also to sample selection and the choice of reference method. We have avoided selection bias in our sensitivity evaluation by using samples from consecutive patients with a positive heterophile antibody test. In this way none of the three EBV IgM assays under evaluation was privileged, and we believe that in this way a correct comparison of the three methods is possible. Classically, indirect immunofluorescence is used as a reference method in EBV serology, but in fact there is no real “gold standard” in EBV serology. The main advantages of immunoblotting as a confirmatory method are the ability to show IgM reactivity to different isolated and specific antigens from the virus and the possibility to objectively evaluate in a standardized way the final results.

EBV serology is performed in primary care mainly to differentiate acute EBV infections from other acute diseases with similar clinical presentations. From our data it is clear that this differentiation cannot be made by performing an isolated EBV IgM test using one of the three immunoassays with their current performance. Assuming a specificity of 98% and a sensitivity of 89%, but considering the very low frequency of possible acute cases (2.2%) as we observed in our prospective study, the positive predictive value of an isolated EBV IgM determination would only be 55.6%. Moreover, CMV, a frequent cause of mononucleosilike illnesses, produces a very high number of positive EBV IgM results, not only in the three assays under evaluation but also on immunoblotting. Positive EBV IgM results caused by other acute infectious illnesses have been described frequently and are usually attributed to either polyclonal B-cell stimulation, cross-reactivity of the IgM antibodies, nonspecific reactivity (natural antibodies), or EBV viral reactivation (1, 13, 16, 17). Discerning the exact cause of the positive EBV IgM result can be very difficult and requires complementary testing using other serological markers and follow-up samples to evaluate the serological evolution. Quantitative EBV viral load determination might also provide useful information in this situation, especially in the case of a possible EBV viral reactivation, but the results should be interpreted cautiously, and this method should be reserved for selected cases (9, 10, 15). In daily practice, however, to overcome these shortcomings, positive EBV IgM results should be complemented with EBNA IgG determinations.

Assuming a specificity of 98% and a sensitivity of 89%, the negative predictive value of an isolated EBV IgM determination would be 99.7%. This means that in our laboratory, a negative EBV IgM result almost excludes an acute EBV infection. On the other hand, all three methods missed at least five cases (7.8%) in the heterophile antibody-positive group due to the lack of EBV IgM antibodies targeted against the VCA p18-p19 antigen, which is used in all three EBV VCA IgM assays. These five samples were clearly positive on immunoblotting, with VCA p19 being negative but VCA gp125 being positive. Implementing an extra antigen (e.g., gp125) in the assays would improve their sensitivity.

As already mentioned by Gärtner et al., standardization of serological profiles (i.e., recent, transient, convalescent, etc.) is missing (8). In the prospective part of our study we used a very simple interpretation scheme in which the majority of cases could easily be classified. Focusing on the possible recent infections in this prospective part, the results are rather disappointing: of eleven cases where one of the three methods suggested a recent infection, in only one case was there a consensus between the three methods. It is clear that determining the exact serologic EBV status on one sample is sometimes not possible, and a follow-up sample should be taken in case of a possible recent infection.

In March 2009 we showed in an earlier study that acute parvovirus B19 infection frequently causes false-positive EBV IgM results on Liaison (2). From the present study, in which we used some of the samples that we used in the previous study, it is clear that the EBV IgM assay on Liaison has much improved: 11 samples of 15 were positive with EBV IgM lot 047 (used in the previous study) but were negative with EBV IgM lot 052 (used in the present study). This improvement of mainly assay specificity has recently also been noticed by Costa et al. (5). However, acute rubella infections, also known for

| Study ID | Liaison | Immulite | BioPlex |
|----------|---------|----------|---------|
|          | VCA IgM | VCA IgG  | EBNA IgG| VCA IgM | VCA IgG  | EBNA IgG| VCA IgM | VCA IgG  | EBNA IgG|
| 1116     | >160 (+) | <10 (–)  | <3 (–)  | 11.9 (+) | 1.3 (+)  | 0.4 (–) | >4.0 (+) | 0.2 (–)  | 0.2 (–)  |
| 1187     | >160 (+) | >750 (+) | <3 (–)  | 7.2 (+)  | ND       | 4.2 (+) | 4.0 (+)  | 0.6 (–)  | 0.2 (–)  |
| 1215     | >160 (+) | >66 (–)  | <3 (–)  | 7.7 (+)  | ND       | 1.2 (+) | >4.0 (+) | 0.2 (–)  | 0.2 (–)  |
| 1225     | 53 (+)   | 135 (–)  | 6 (–)   | 0.2 (–)  | ND       | 28.0 (+) | 0.7 (–)  | ND       | 8.0 (–)  |
| 1244     | 56 (+)   | <10 (–)  | 6 (–)   | 0.4 (+)  | 0.2 (+)  | 0.4 (–) | 2.1 (+)  | 0.2 (–)  | 0.2 (–)  |
| 1262     | 71 (+)   | ND       | 29 (+)  | 3.7 (+)  | 26.0 (+) | 0.7 (–) | 1.8 (+)  | ND       | 1.5 (+)  |
| 1297     | 105 (+)  | 56 (–)   | <3 (–)  | 0.4 (–)  | 7.3 (+)  | 0.5 (–) | 0.4 (–)  | ND       | 1.5 (+)  |
| 1309     | 62 (+)   | 99 (–)   | 3 (–)   | 0.7 (–)  | 12.9 (+) | 0.2 (–) | 2.5 (+)  | 0.4 (–)  | 0.2 (–)  |
| 1397     | 80 (+)   | 530 (–)  | <3 (–)  | 1.9 (+)  | ND       | 25.9 (+) | 4.0 (+)  | ND       | 4.9 (–)  |
| 1408     | 40 (+)   | 51 (–)   | <3 (–)  | 1.1 (+)  | 11.3 (+) | 0.3 (–) | 0.5 (–)  | 2.0 (+)  | 0.2 (–)  |
| 1470     | 34 (–)   | 130 (–)  | <3 (–)  | 0.4 (+)  | 18.2 (+) | 1.0 (–) | 1.3 (+)  | 4.7 (+)  | 0.5 (–)  |

* Values are expressed as mU/liter for Liaison and as ratios for BioPlex and Immulite. The applied cutoffs for the assays on Liaison were 40 mU/liter for VCA IgM, 20 mU/liter for VCA IgG, and 20 mU/liter for EBNA IgG. For the assays performed on BioPlex and Immulite, a cutoff 1.1 (ratio) was used. The result interpretation as positive or negative is indicated in parentheses.

* a ND, not determined.
causing false-positive EBV IgM results on Liaison, remain a persistent problem, since 6 of the 14 cases (43%) we examined here were false positive on Liaison, whereas with BioPlex and Immulite only 1 false-positive case was seen. The false-positive EBV IgM results on Liaison due to acute rubella virus infections can be eliminated by adding extra blocking reagents to the assay buffer, such as polyvinylpyrrolidone and polyvinyl alcohol or 10% Roti-Block (Carl Roth, Karlsruhe, Germany) (2, 3; unpublished observations).

The very slow or absent VCA IgG seroconversion as seen on BioPlex, compared to Liaison and Immulite, can be a drawback of the BioPlex system. Waiting several weeks to months before a significant VCA IgG titer change or VCA IgG seroconversion can be shown could have a significant impact on patient care. This apparent low sensitivity of the VCA IgG on BioPlex in acute EBV infections was also illustrated by the very low prevalence of VCA IgG-positive results in the heterophile antibody-positive group. Since our study was not designed to evaluate the VCA IgG performance on the three platforms, and an important selection bias was present in this part of the study, further studies need to be performed to clarify this possible assay problem.

In conclusion, we can say that the three EBV IgM immunoassays we evaluated have an acceptable and comparable performance, but there remains room for improvement. For the diagnostic work-up of infectious mononucleosis or mononucleosis-like illnesses in primary care, a negative EBV IgM almost excludes an acute EBV infection, but a positive EBV IgM test should be cautiously interpreted.

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