Comparison of two automated instruments for Epstein–Barr virus serology in a large adult hospital and implementation of an Epstein–Barr virus nuclear antigen-based testing algorithm

Hilal Al Sidairi, Khalifa Binkhamis, Colleen Jackson, Catherine Roberts, Charles Heinsein, Jimmy MacDonald, Robert Needle, Todd F. Hatchette, and Jason J. LeBlanc

Abstract

Serology remains the mainstay for diagnosis of Epstein–Barr virus (EBV) infection. This study compared two automated platforms (BioPlex 2200 and Architect i2000SR) to test three EBV serological markers: viral capsid antigen (VCA) immunoglobulins of class M (IgM), VCA immunoglobulins of class G (IgG) and EBV nuclear antigen-1 (EBNA-1) IgG. Using sera from 65 patients at various stages of EBV disease, BioPlex demonstrated near-perfect agreement for all EBV markers compared to a consensus reference. The agreement for Architect was near-perfect for VCA IgG and EBNA-1 IgG, and substantial for VCA IgM despite five equivocal results. Since the majority of testing in our hospital was from adults with EBNA-1 IgG positive results, post-implementation analysis of an EBNA-based algorithm showed advantages over parallel testing of the three serologic markers. This small verification demonstrated that both automated systems for EBV serology had good performance for all EBV markers, and an EBNA-based testing algorithm is ideal for an adult hospital.

Epstein–Barr virus (EBV) infects nearly every human by adulthood [1, 2]. EBV transmission mainly occurs through saliva or sexual contact, and peak occurrences are seen in children and adolescents [3–8]. While the spectrum of EBV diseases includes lymphoproliferative disorders, primary infections are mainly asymptomatic or present as infectious mononucleosis (glandular fever) [2–5]. Treatment of infectious mononucleosis is mainly supportive, and serology is the mainstay for diagnosis [1, 9–12]. This study compared the performance of two random access, automated instruments for the detection of three EBV serological markers.

Traditionally, the mononuclear spot test (or monospot test) was often used for detection of infectious mononucleosis based on the presence of heterophile antibodies in patients with EBV disease [1, 9, 11]. Monospot testing is often part of routine diagnostic testing algorithms; however, this test is manual, the performance varies with age, and the results are subjective and not specific for EBV disease [1, 9–12]. Rather than being specific to the immune response to EBV, heterophile antibodies are directed at antigens that allow agglutination of cells of other animal species [1]. As such, monospot testing is no longer recommended [11]. Today, EBV-specific serology including immunoglobulins of class M (IgM) and G (IgG) against EBV viral capsid antigen (VCA), and EBV nuclear antigen-1 (EBNA-1) IgG, are widely accepted serological markers for EBV infection in immune-competent patients [1, 9–12]. VCA IgM is a marker of acute infection, and usually disappears within 4–6 weeks of infection [11]. VCA IgG appears in the acute phase of disease, peaks at 2–4 weeks after the onset of symptoms, and persists for life [11]. Although there can be a short overlap between the disappearance of anti-VCA IgM and the appearance of EBNA-1 IgG, this antibody is not seen in acute EBV infection, and EBNA-1 IgG is a considered marker of a past infection [1, 11]. It appears within 2–4 months after the onset of symptoms, and persists for life [1, 11]. Altogether, these markers can help identify recent or past exposures to EBV [1, 9–12].

Laboratory methods for EBV-specific serology have evolved from manual immunofluorescence assay (IFA) and enzyme

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Author affiliations: 1Division of Microbiology, Department of Pathology and Laboratory Medicine, Nova Scotia Health Authority (NSHA), Halifax, NS, Canada; 2Dalhousie University, Halifax, NS, Canada; 3King Saud University, Riyadh, Saudi Arabia; 4Public Health & Microbiology Laboratory, Eastern Health, St. John’s, NL, Canada.

*Correspondence: Jason J. LeBlanc, jason.leblanc@nshealth.ca

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Abbreviations: CI, confidence interval; CMIA, chemiluminescent microparticle immunoassays; EA-D, early antigen-diffuse; EBNA-1, EBV nuclear antigen-1; EBV, Epstein–Barr virus; EIA, enzyme immunoassay; HSC, Health Sciences Center; IFA, immunofluorescence assay; IgG, immunoglobulins of class G; IgM, immunoglobulins of class M; MFI, multiplex flow immunoassays; QEII, Queen Elizabeth II; VCA, viral capsid antigen.

Two supplementary tables are available with the online Supplementary Material.
immunoassay (EIA) methods, to chemiluminescent microparticle immunoassays (CMIA) and multiplex flow immunoassays (MFI) [11–21]. CMIA- and MFI-based assays on automated instruments have streamlined EBV serologic testing, and displayed excellent agreement with traditional methods [11–21]. However, to date, the performance of EBV panels using CMIA on the Abbott Architect or MFI on the BioPlex 2200 instruments have not yet been directly compared. Since the presence or absence of different EBV antibodies is dynamic and varies with the stage of EBV disease [1, 20–23], this study compared the performance of VCA IgM, VCA IgG and EBNA-1 IgG detection assays on the BioPlex and Architect-automated instruments using two different testing algorithms: (1) an algorithm based on testing all three markers simultaneously; and (2) an algorithm where EBNA-1 IgG is tested first, and if positive, precludes testing for VCA IgM and IgG [20, 22].

For instrument comparison, anonymized sera unlinked to clinical information were provided as a blind panel by the Public Health Laboratory and Microbiology, Eastern Health (St. John’s, NL). The sera were selected randomly from archived specimens that had previously been tested using the anti-EBV-VCA IgM, anti-EBV-VCA IgG and anti-EBNA-1 IgG ELISA kits as per manufacturer recommendations on a Euroimmun Analyzer 1-2P (Medizinische Labordiagnostika, Luebek, Germany). The sera test results were obtained from 65 patients with various serologic profiles, which included results for VCA IgM (18 positive; 47 negative), VCA IgG (46 positive; 19 negative) and EBNA-1 IgG (36 positive; 29 negative). All sera were stored as aliquots at −20 °C until use, and were subjected to no more than one freeze–thaw cycle. The comparison of the BioPlex 2200 (Bio-Rad Laboratories, Mississauga, ON) and Architect i2000SR (Abbott Laboratories, Diagnostics Division, Abbott Park, IL) was performed at the Queen Elizabeth II (QEI) Health Sciences Center (HSC) in Halifax Nova Scotia, Canada. All sera were tested in parallel using the EBV IgM and IgG kits on a BioPlex 2200, or the EBV VCA IgM, EBV VCA IgG and EBV EBNA-1 IgG kits on the Architect i2000SR. All tests were calibrated, performed and interpreted according to manufacturer recommendations.

Due to the lack of a reference method for EBV infection, results from each serological marker (VCA IgM, VCA IgG or EBNA-1 IgG) obtained from each automated method were compared to a consensus defined as two of three concordant results between those obtained from the automated instrument (BioPlex or Architect), those obtained by the Euroimmun assay, and a third test that was used to resolve discrepant results between these two if needed. The discrepant analyses used indirect IFA with the Merifluor EBV IgM IFA kit (Meridian BioScience, Cincinnati, OH) for VCA IgM, and for VCA IgG and EBNA-1 IgG, the ELISA EBV VCA IgG and the ELISA EBNA-1 IgG kits (Zeus Scientific, Branchburg, NJ) were performed on a DS2 ELISA Automation System (Dynex Technologies, Chantilly, VA). The relative sensitivity and specificity of the BioPlex and Architect assays were calculated with 95 % confidence intervals (CIs) for each EBV marker. The level of agreement between each method and the consensus was determined using kappa coefficients with defined values as follows: poor (from 0 to 0.2), fair (from 0.21 to 0.4), moderate (from 0.41 to 0.6), substantial (from 0.61 to 0.8), or near-perfect (from 0.81 to 1.0) [24]. Consistent with previous reports, the grey zone or equivocal results were considered negative during the relative sensitivity and specificity analyses [14–20]. Statistical analyses were performed using Statistical Analysis Software (SAS) version 9.4 (SAS Institute, Cary NC).

In this study, near-perfect agreement between BioPlex or the Architect EBV assays was seen when compared to a consensus. The BioPlex results showed 100 % relative sensitivity and specificity for both VCA IgG and EBNA-1 IgG, and only one discrepant positive result was noted for VCA IgM (Table 1). In this case, the discrepant positive would have little impact as the case would have been classified as a late primary infection, as opposed to a past infection (Table 2).

### Table 1. Comparison of the BioPlex and Architect EBV serology panels

<table>
<thead>
<tr>
<th>Testing algorithm</th>
<th>Test result</th>
<th>Instrument</th>
<th>Relative % sensitivity (95 % CI)</th>
<th>Relative % specificity (95 % CI)</th>
<th>Kappa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parallel</td>
<td>VCA IgM</td>
<td>BioPlex</td>
<td>100.0 (78.1 to 100.0)</td>
<td>97.8 (87.2 to 99.9)</td>
<td>0.96 (0.89 to 1.00)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Architect</td>
<td>72.2 (46.4 to 89.3)</td>
<td>97.4 (87.2 to 99.9)</td>
<td>0.75 (0.56 to 0.94)</td>
</tr>
<tr>
<td></td>
<td>VCA IgG</td>
<td>BioPlex</td>
<td>100.0 (90.3 to 100.0)</td>
<td>100.0 (79.1 to 100.0)</td>
<td>1.00 (1.00 to 1.00)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Architect</td>
<td>100.0 (90.3 to 100.0)</td>
<td>94.7 (71.9 to 99.7)</td>
<td>0.96 (0.89 to 1.00)</td>
</tr>
<tr>
<td></td>
<td>EBNA-1 IgG</td>
<td>BioPlex</td>
<td>100.0 (89.0 to 100.0)</td>
<td>100.0 (85.4 to 100.0)</td>
<td>1.00 (1.00 to 1.00)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Architect</td>
<td>97.2 (83.8 to 99.8)</td>
<td>100.0 (85.4 to 100.0)</td>
<td>0.97 (0.91 to 1.00)</td>
</tr>
<tr>
<td>EBNA-based</td>
<td>VCA IgM</td>
<td>BioPlex</td>
<td>100.0 (79.1 to 100.0)</td>
<td>100.0 (90.3 to 100.0)</td>
<td>1.00 (1.00 to 1.00)</td>
</tr>
<tr>
<td></td>
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<td>Architect</td>
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</table>
samples submitted to laboratories originating from adult patient populations with past infections [12], algorithms have been used where EBNA-1 IgG would preclude testing for VCA IgM and VCA IgG (unless specifically requested), whereas EBNA-1 IgG negative or equivocal results would prompt VCA IgM and IgG testing [20, 22]. If tested in an EBNA-based algorithm, the discrepant positive VCA IgM on BioPlex could have been avoided (Tables 1 and S1, available in the online Supplementary Material). Overall, this study showed near-perfect agreement between BioPlex and the consensus for all serological markers in every stage of EBV disease, which is in agreement with the manufacturer’s claims.

The performance is consistent whether tested in parallel or in an EBNA-based algorithm (Tables 1, 2 and S1). For intra-run and inter-run reproducibility on the BioPlex, five samples spanning low, moderate and high positive specimens for each EBV marker (VCA IgM, VCA IgG and EBNA-1) was assessed in triplicate within experiments, and replicates of the same specimens were tested over five independent runs. The percentage coefficients of variation were all less than 2.0 % and consistent with manufacturer recommendations. Overall, the BioPlex displayed excellent performance characteristics; however, this does not preclude the possibility of discrepant positive or negative results for any of the EBV serological markers, nor should these results be extrapolated to serological testing outside the diagnosis of infectious mononucleosis in a primarily adult population.

For Architect, results similar to those of BioPlex were obtained for VCA IgG and EBNA-1, with the exception of one discrepant positive VCA IgG and one discrepant negative EBNA-1 IgG (Table 1). When repeated, no changes were noted in the test results. First, the discrepant reactive VCA IgG had a signal of 1.10, which is near the cutoff for positivity of ≥1.0. For this case, all other EBV serological markers performed on the Architect and BioPlex, as well as the Euroimmun and Zeus EIAs, were all negative. Therefore, this discrepant positive VCA IgG misclassified the case as an isolated VCA IgG rather than seronegative (Table 2). Second, the discrepant negative EBNA-1 IgG result with Architect was seen in a patient where positive results were obtained with BioPlex and the Euroimmun and Zeus EBNA-1 IgG EIAs. This would also misclassify the results as an isolated VCA IgG instead of a past infection (Table 2). Both the discrepant positive VCA IgG and one discrepant negative EBNA-1 IgG would have little impact in an immune-competent host, but these scenarios could have serious implications in the setting of pre-transplant screening where individuals could be at risk of a recipient/donor mismatch [1].

VCA IgM results on Architect also showed discrepant results that were reproducible upon repeat testing (Table 1). Like BioPlex, Architect displayed a discrepant positive VCA IgM that would have staged the disease as a late primary

| Table 2. Comparison of BioPlex and Architect EBV panels |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| VCA IgM/EBNA-1 IgG | Serological profile | BioPlex EBV panels | Architect EBV panels |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Seronegative | Early acute primary infection | Acute primary infection | Late primary infection (or reactivation) | Past infection | Isolated VCA IgG | Isolated EBNA-1 IgG |
| BioPlex EBV panels | Seronegative | 13 | 0 | 0 | 0 | 0 | 0 | 0 |
| +/+/+ | Late primary infection (or reactivation) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| --/+ | Past infection | 0 | 0 | 0 | 0 | 29 | 0 | 0 |
| --/+ | Isolated VCA IgG | 0 | 0 | 0 | 0 | 0 | 0 | 40 |
| --/+ | Isolated EBNA-1 IgG | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Total | 13 | 6 | 6 | 6 | 30 | 4 | 0 | 0 |
| Architect EBV panels | Seronegative | 12 | 1* | 0 | 0 | 0 | 0 | 0 |
| +/+/+ | Late primary infection (or reactivation) | 0 | 0 | 0 | 0 | 2 | 1* | 0 |
| --/+ | Past infection | 0 | 0 | 0 | 4* | 28 | 0 | 0 |
| --/+ | Isolated VCA IgG | 1* | 0 | 0 | 0 | 0 | 0 | 4 |
| --/+ | Isolated EBNA-1 IgG | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Total | 13 | 6 | 6 | 6 | 30 | 4 | 0 | 0 |

*Discordant result between BioPlex or Architect compared against the consensus.
infection as opposed to past infection with little impact. In addition, there were five positive VCA IgM results by the consensus that fell within the Architect equivocal range (Table 1). Four of the five discrepant negative results had little impact, as they classified late primary infections as past infections. The last equivocal result was considered a discrepant negative VCA IgM and missed an early acute primary infection (Table 2). Overall, the agreement between Architect and the consensus was near-perfect for VCA IgG and EBNA-1 IgG, and remained moderate to substantial for VCA IgM despite the decreased relative sensitivity attributed to the five equivocal results. If the equivocal results for VCA IgM were considered positive, or when testing was performed as part of an EBNA-based algorithm, the agreement with the consensus was near-perfect (Tables 1 and S1). For equivocal results, it would be prudent to repeat EBV serology after 1–2 weeks or submit the specimen for testing using an alternative method [1].

Both BioPlex and Architect are high throughput random access instruments that offer many advantages compared to traditional IFA- or EIA-based serologic testing. In contrast to testing in batches as seen with EIA, random access on BioPlex allows for high throughput testing of individual patient specimens. The use of automated instruments for EBV serology can streamline testing, and facilitate implementation of cost-effective testing algorithms for specific patient populations [17, 20, 22]. In a recent study where 81% of the population was EBNA-1 IgG positive, the EBNA-based testing algorithm using Architect reduced the number of tests required by approximately 50% compared to testing all three serologic markers in parallel, without compromising the assay abilities to stage EBV infections [20]. In this study, the distribution of EBNA-1 IgG results was assessed following implementation of BioPlex 2200 at the QEII HSC (Halifax, NS) on 18 January 2016 and 1 June 2017 (Fig. 1, Table S2). In this time period, 69.4% (3614/5205) of cases were identified as EBNA-1 IgG positive (Table S2) and the age distribution of EBNA-1 IgG positive patients is consistent with an adult hospital (Fig. 1). Compared to individuals aged 16 or younger, adults over the age of 16 had a high proportion of EBNA-1 IgG positive results (74.0% vs 30.3%), a lower proportion of seronegative cases (13.2% vs 54.3%), and less early acute infections (5.1% vs 9.4%) (Fig. 1b, Table S2). Of note, the distribution of serologic profiles for children (defined as individuals aged 16 or under) or adults over the age of 70 years were underrepresented, which may have led to sampling errors in the proportions of positive EBNA-1 IgG results for individuals in these categories (Fig. 1b). Regardless, the EBNA-1 results clearly demonstrated a high proportion of positive results, reaching over 85% in all age groups above 30 years (Fig. 1).

The EBNA-based algorithm on BioPlex 2200 streamlined EBV serology testing and reduced testing numbers by 46.3% compared to parallel testing for each of the three EBV markers (Table S2). For BioPlex testing, there are two different reagent kits which need to be loaded on the instrument: the EBV IgM panel that includes VCA-IgM and heterophile antibody tests, and the EBV IgG kit that detects VCA IgG, EBNA-1 IgG and early antigen-diffuse (EA-D) IgG. In our institution, the vendor contract for EBV testing on BioPlex was issued as a cost per reportable and the cost for each EBV marker was priced at $3.50 CAD. As such, the cost for the 15 615 tests that would have incurred with parallel testing of each marker would have cumulated to $54625.50 CAD. In the EBNA-based algorithm, only a subset of testing was performed (5205 EBNA-1 IgG, and 1591 tests each for VCA IgM and VCA IgG), which amounted to $29354.50 CAD. In other words, the EBNA-based algorithm led to a cost saving of approximately $25271.00 CAD in reagent costs alone. In addition, since the EBNA-based algorithm precludes the need for VCA IgM testing in EBNA-1 positive patients, it reduces the possibility of discrepant positive VCA IgM results known to occur from the presence of rheumatoid factor, auto-antibodies or cross-reacting factors during human cytomegalovirus or parvovirus B19 infections [25]. During the validation period, these benefits were demonstrated on the BioPlex platform, with near-perfect agreement compared to the consensus with using the EBNA-1 IgG-based algorithm (Table 1).

While the EBNA-based algorithm is cost-effective in an adult population and helps avoid discrepant reactions with VCA IgM and IgG, it should be noted that unless the laboratory is notified, VCA IgM and VCA IgG testing may not be performed following the EBNA-1 IgG positive results. As such, patient results characterized as late primary infection or reactivation would be classified as a past infection (Table S1). This would have little impact in immunocompetent adults [1, 20, 25]. The cost benefits of the EBNA-based algorithm, with its 46% reduction in testing numbers, outweighs the need for education regarding the subtle caveats of this testing approach in a predominantly EBNA-1 IgG patient population (Fig. 1, Table S2). For immunocompromised individuals or patients with hematologic malignancies, EBV serology itself can be misleading regardless of the testing algorithm, and EBV disease can be monitored using techniques like quantitative PCR or in situ hybridization from tissue biopsy [25–27]. While serologic approaches are useful for infectious mononucleosis, applying them outside this clinical setting could give rise to misleading results.

It should also be noted that automation cannot address all the problems associated with interpretations of EBV serology. Discrepant positive or negative reactions could occur with any EBV marker, and unresolved serologic profiles have been observed in up to 10% of cases due to equivocal (or indeterminate) results, interfering substances or isolated reactive results for VCA IgM, VCA IgG or EBNA-1 IgG [1, 1–21, 28, 29]. In this study, the EBNA-based algorithm provided results for 96.8% of patients, with only a subset (3.3%) that remained inconclusive due to unresolved serologic profiles that included isolated VCA IgG results (2.5%), equivocal results (0.7%) and interfering substances (0.1%) (Table S2). While some studies suggested that
resolution may require additional testing like avidity testing, immunoblot analyses, or qualitative/quantitative EBV PCR [9, 25–34], the clinical context of each patient should be considered [19], and resolution can often be achieved with serial EBV serology testing over time. These concepts should be considered in a disclaimer on laboratory reports.

Compared to Architect, MFI-based assays on BioPlex further simplify the workflow [18, 19]. With its multiplexing technology using fluorescent colour-coded beads coupled to a capture system for the antibodies of interest, each sample is queried for various target antibodies in a single reaction. The instrument can both identify positive results and discriminate which antibody or antibodies were present, and as such, the BioPlex assays allow the simultaneous detection of multiple serologic markers within one experiment. In addition to the three EBV markers evaluated in this study, the BioPlex EBV panels also include the detection of heterophile IgM antibodies and EA-D IgG. During validation, a heterophile IgM antibody was only detected in three/six cases of acute EBV infection and two/six cases of late primary infection, whereas EA-D IgG was present in four/six cases of early acute primary infection, four/six cases of acute primary infection, one case of late primary infection, and four/thirty cases of past EBV infection (data not shown). While testing for the heterophile antibody or EA-D IgG is controversial, the data from this study is consistent with the notion that these markers have limited value for staging EBV disease [1, 11, 13, 18–20]. With possible cross-reactions which are known to occur with heterophile IgM antibodies [1], and misclassifications attributed to EA-D IgG

Fig. 1. Percentage of EBNA-1 IgG positive results by age. (a) Distribution of the percentage of positive EBNA-1 IgG results by age. The solid line represents a trend line for the average. (b) Proportion of EBNA-1 IgG results categorized by age. All data was obtained on BioPlex 2200.
[18, 19], serologic testing for EBV in our adult hospital was limited to VCA IgM, VCA IgG and EBNA-1 IgG.

Overall, this study conducted the first direct comparison between the performance of EBV assays on BioPlex and Architect. Using an EBNA-based algorithm and cutoff for concordance of approximately 95% against the consensus, both automated platforms had acceptable performance for identification of VCA IgM, VCA IgG and EBNA-1 IgG. However, unlike the Architect assays, the BioPlex assays did not show any discrepant results compared to the consensus. While the verification of the assays was limited by the small number of pre-selected sera spanning different serologic profiles, the numbers for each EBV marker were sufficient to compare the overall performance of the instruments, which themselves had previously been validated against other automated assays, ELAs and IFAs [13–21]. While it could be argued there is a high cost for instrumentation, many hospitals routinely use BioPlex or Architect instruments for infectious diseases and other diagnostic testing. At our adult hospital, both Architect and BioPlex instruments were readily available, which enabled us to transition away from antiquated serologic testing based on heterophile antibodies and manual ELAs. With 70% of our patient population being EBNA-1 IgG positive, the validity of an EBNA-based algorithm was justified, and has been shown to be a cost-effective approach compared to parallel testing of EBV markers.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
Testing for validation and prospective analyses were performed for quality assurance purposes using anonymized samples and data following local REB approval processes.

References


