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Comparison of a multiplexed bead-based assay with an immunofluorescence and an enzyme-immuno assay for the assessment of Epstein–Barr virus serologic status

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Abstract

We have compared a multiplexed bead-based assay (BBA) with an enzyme immunoassay (EIA) and immunofluorescence assay (IFA) for the assessment of the Epstein–Barr virus (EBV) serostatus. Three hundred and ninety-three sera, classified according to IFA results as seronegative (n = 100), acute infection (n = 100), past infection (n = 100) and indeterminate (n = 93), were tested by BBA and EIA. Overall, the three methods gave similar results with a relatively high (75.2%) concordance with the consensus interpretation of the serostatus. The most significant discordances were: (i) 58 samples had uninterpretable results for BBA, in majority due to the detection of non-antigen specific antibody binding by control beads. (ii) almost half the samples positive for anti-Epstein–Barr nuclear antigen (EBNA) IgG by BBA or EIA were negative by IFA. Among the latter, only a minority had a history of immunocompromise or treatment, or detectable anti-early antigen antibody. This discrepancy probably reflects a poor sensitivity of IFA for anti-EBNA IgG detection. EIA and BBA had a similar performance and had substantial practical advantages over IFA with respect to testing for EBV serostatus.

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Introduction

Epstein–Barr virus (EBV) is a gamma-herpesvirus that transmits readily in humans, mostly by the oral route, infecting more than 95% of the population worldwide [1]. After a primary infection, either asymptomatic or manifesting as an infectious mononucleosis and characterized by viral replication in the oropharynx and in B lymphocytes, EBV-specific T cells responses curtail viral replication. The virus DNA then persists as an episomal DNA genome in memory B lymphocytes, with a very minimal gene expression, described as a latent infection. Latent infection is related to lymphoproliferative and other malignant diseases in a complex manner [2].

The diagnosis of the various stages of EBV infection and of EBV-related malignant conditions is partly based on the detection of different classes of antibodies specific for various EBV antigens [the lytic viral capsid antigens (VCAs), the latency-associated Epstein–Barr nuclear antigens (EBNAs) and the replicative early antigens (EAs)] [3]. Multiplexed assays offer the opportunity to assess antibody responses to a panel of antigens in a single, specimen-sparing assay, with reduced time requirements. We therefore compared the diagnostic performance of the Athena Multi-Lyte® multiplexed bead-based assay (BBA) for the detection of EBV-specific antibodies with the monoplexed immunofluorescence assay (IFA) and enzyme immunoassay (EIA).

Materials and Methods

Patient samples

A total of 393 serum samples received in the serology laboratory of the Institute of Microbiology, Centre Hospitalier Universitaire Vaudois, for EBV testing by IFA between 1998

and 2005 were included in the study. The sample population included adult and paediatric specimens and a mix of specimens from immunocompromised (e.g. solid organ transplant) patients and those suspected of acute or latent EBV infection.

The serum samples were classified into five groups according to their IFA serostatus pattern: (i) 100 non-infected (seronegative, anti-VCA IgM–, anti-VCA IgG– and anti-EBNA IgG–); (ii) 100 with acute infection (anti-VCA IgM+, anti-VCA IgG+/– and anti-EBNA IgG–, only 94 of which were available for analysis by multiplexed BBA); (iii) 100 with past infection (anti-VCA IgM–, anti-VCA IgG+ and anti-EBNA IgG+); (iv) 70 with type I indeterminate pattern, possibly immunocompromised patients who had lost or not developed responses against EBNAs or, rarely, acute infection in the absence of anti-VCA IgM (anti-VCA IgM–, anti-VCA IgG+ and anti-EBNA IgG–); (v) 23 with type II indeterminate pattern, possibly sub-acute infection or reactivation (anti-VCA IgM+, anti-VCA IgG+ and anti-EBNA IgG+). The samples had been stored in the temperature range −20° to −40°C from the time of IFA testing and were centrifuged for 5 min at 8000 ***g*** to remove protein aggregates after thawing before testing by multiplexed BBA and EIA.

EBV-specific antibody testing

For each serum, the antibodies assayed were: anti-VCA IgM, anti-VCA IgG and anti-EBNA IgG. The anti-EA IgG was tested only in the BBA.

The serum samples had initially been analyzed by IFA (Merifluor®), anti-EBV IgM and IgG IFA/IFT (Meridian Bioscience, Cincinnati, OH, USA) and anti-EBNA ACIF (Focus Technologies, Herndon, VA, USA), in accordance with the manufacturers' instructions [4]. Sixty microliters of serum were used to analyse the three antibodies. HR1 cells expressing the VCA antigen were used in the anti-VCA IgG and IgM test kit [3,4]. According to the manufacturer, the antigen used in the anti-EBNA IgG kit comprises 'lymphoid cells chosen for their selective production of EBNA antigen'. Anti-VCA IgM was tested at a single 1 : 10 dilution in IFA and samples were arbitrarily assigned a 1 : 5 titre if negative and 1 : 20 if positive.

The samples were then analyzed by EIA (Novitec® EBV-EIA; Genbio, San Diego, CA, USA) and BBAs were performed on a Luminex 100 reader (Athena Multi-Lyte EBV IgG and IgM; Zeus Scientific, Raritan, NJ, USA), in accordance with the manufacturer's instructions. In the EIA, 10 μL of serum were diluted and used to analyse the three antibodies for each sample. The VCA antigen comprised affinity-purified gp125 VCA from a glycine extract of lysates of EBV-infected cells (IgG and IgM) in EIA and BBA, whereas the EBNA antigen was a recombinant EBNA-1 expressed in a baculovirus system for EIA and expressed in *Escherichia coli* for BBA. In the BBA, 10 μL serum were used for the three IgG assays and 10 μL were used for the IgM test. The BBA has built-in controls that assess the binding of antibodies to beads not coated with antigen (nonspecific coating; NSC), to minimize the false positive results.

Rheumatoid factor

To test the effect of rheumatoid factor on the results of anti-VCA IgM by BBA, 46 prospective routine sera that gave an NSC alarm (see above) for IgM were studied. Rheumatoid factor was assayed by nephelometry using the N Latex RF kit (Dade Behring, Eschborn, Germany). In addition, 10 μL of serum were diluted in 200 μL of sample diluent containing Fc-specific anti-IgG goat antiserum (Sample diluent 005M; Zeus Scientific) and centrifuged for 5 min at 8000 ***g*** to remove IgG. The supernatant was used in the BBA as described above.

Serostatus interpretation

For each method, the dilution or index value was translated into positive, negative or indeterminate qualitative results, in accordance with the manufacturer's instructions.

To compare the diagnostic interpretations of the three methods, a consensus interpretation was established for each antibody response using a majority rule. The consensus was defined as the majority interpretation if two out of three or three out of three assays produced the same antibody interpretation. There was therefore no defined consensus when all three results were different (or when one of the assays produced an uninterpretable result; e.g. when the BBA gave an undefined result such as NSC and the two others gave discordant results). The anti-EA antigen was available only with the BBA and therefore was not included in this analysis.

The stages of the infection were defined according to the presence of the various antibodies as suggested by Hess [3] (Table 1). Each serum specimen was attributed a serostatus

TABLE 1. Classification of Epstein–Barr virus infection stage according to serostatus pattern

	VCA IgM	VCA IgG	EBNA IgG
Acute infection	+	+/–	–
Past infection	–	+	+
Seronegative	–	–	–
Indeterminate I	–	+	–
Indeterminate II	+	+	+
Non plausible	–	–	+

VCA, viral capsid antigen; EBNA, Epstein–Barr nuclear antigen.

interpretation by each method and by consensus, and each assay serostatus interpretations were compared with the consensus interpretation.

Statistical analysis

The quantitative relationship between antibody titres and indices was evaluated by linear regression using STATA 10 software (StataCorp, College Station, TX, USA) on log-transformed values.

Ethical considerations

The present study was performed using samples left over from clinically motivated EBV testing of patients who had given consent with respect to their potential use for research and development. This procedure was approved by the local research ethics committee.

Results

For each antigen-specific antibody, the results of the three methods were compared (Fig. 1).

Anti-VCA IgM

Fig. 1 (upper left) shows the relationship for anti-VCA IgM assessed by EIA and IFA. There was generally a good qualitative concordance (364/393; 93%) between these two tests when classified as negative/indeterminate/positive. EIA appeared somewhat less sensitive than IFA, with 21 discordant samples (IFA positive/EIA negative). Because IFA testing for anti-VCA IgM was run only qualitatively at a single 1 : 10 dilution, this precluded a quantitative comparison with EIA and BBA indices.

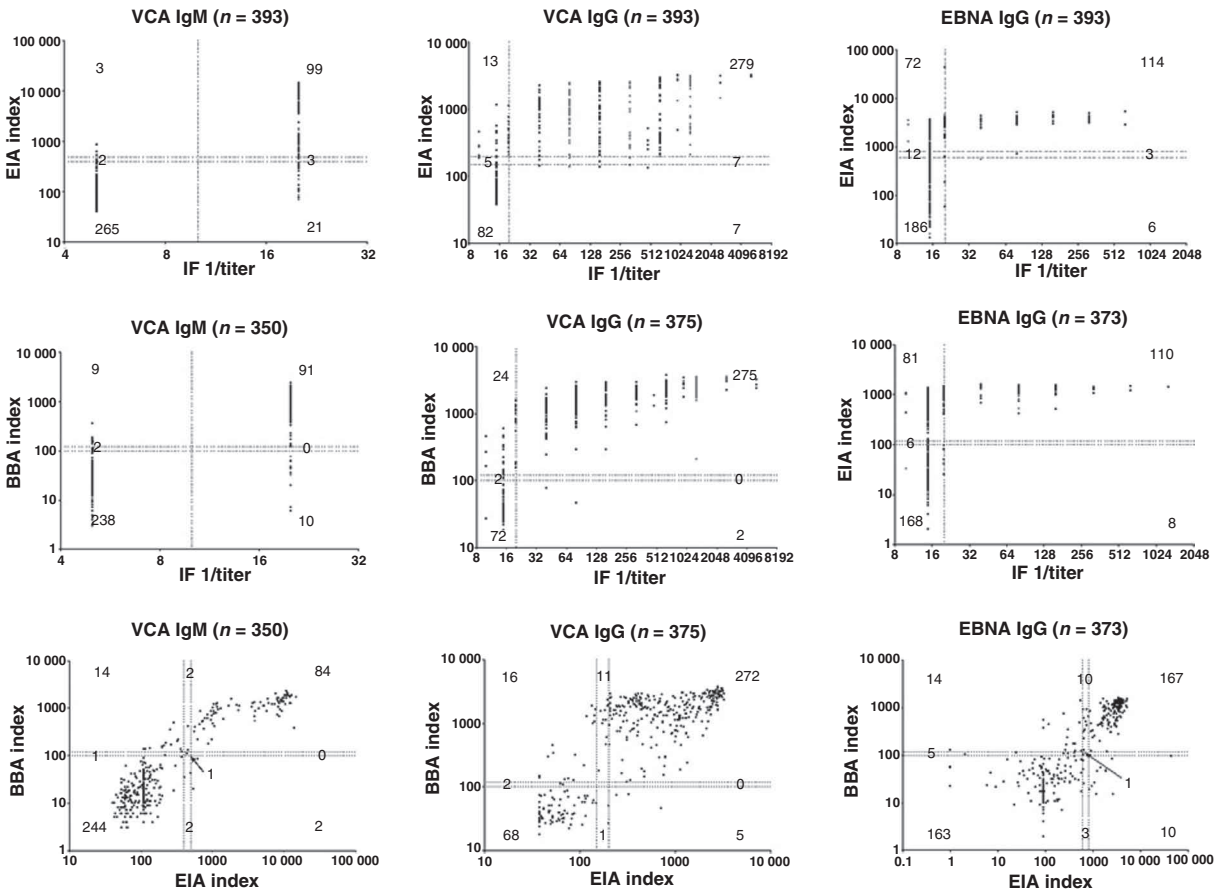

FIG. 1. For each antibody type, the three methods were compared with each other. The lower left (negative) and upper right (positive) quadrants contain concordant data with the corresponding number of sera shown. The upper left and lower right quadrants show discordant data. Numbers of data shown in the grey zone correspond to sera with minor discrepancies. Anti-viral capsid antigen IgM were determined at a single 1 : 10 dilution in the immunofluorescence assay. Samples were arbitrarily assigned a 1 : 5 titre if negative and 1 : 20 if positive. BBA, bead-based assay; EBNA, Epstein–Barr nuclear antigen; EIA, enzyme immunoassay; IF, immunofluorescence; VCA, viral capsid antigen.

Fig. 1 (middle left) shows the relationships of anti-VCA IgM results assessed by BBA and IFA. The smaller number of data points is principally due to invalid NSC results in the BBA. There was a good qualitative concordance (329/350; 94%) between these two tests. BBA had a sensitivity for anti-VCA IgM intermediate between IFA and EIA, with only ten discordant samples (IFA positive/BBA negative).

Finally, Fig. 1 (lower left) shows the qualitative concordance between BBA and EIA indices (328/350; 94%) among the 350 sera for which pairs of results were available. Both EIA and BBA results were expressed as quantitative indices and were highly correlated (r^2 = 0.83).

With the BBA, 46 of 393 samples (among which 36 were NSC for anti-VCA IgM and ten for anti-VCA and anti-EBNA IgG) gave NSC results. The 46 selected sera had detectable rheumatoid factor by nephelometry and gave NSC results for anti-VCA IgM in the initial testing, but had no NSC results when retested after absorption, with 42 of them being negative and four being positive for anti-VCA IgM.

Anti-VCA IgG

Fig. 1 (upper central) shows the relationship between the anti-VCA IgG results obtained with EIA and IFA. There was good qualitative concordance (361/393; 92%) but no quantitative correlation (r^2 = 0.10). Fig. 1 (middle central) panel shows the relationship between the anti-VCA IgG results obtained with BBA and IFA. There was again a good qualitative concordance (347/375; 93%) and a somewhat better quantitative correlation (r^2 = 0.37). Finally, Fig. 1 (lower central) shows the good qualitative concordance between the BBA and EIA results (340/375; 91%) but no quantitative correlation (r^2 = 0.08).

Anti-EBNA IgG

Fig. 1 (upper right) shows the relationship between the anti-EBNA IgG results obtained with EIA and IFA. There was a low qualitative concordance (300/393; 76%) compared to anti-VCA IgM and anti-VCA IgG tests and again no obvious quantitative correlation (r^2 = 0.11). Fig. 1 (middle right) shows the relationship between the anti-EBNA IgG results obtained by BBA and IFA. There was a low qualitative concordance (278/373; 75%) and again no obvious quantitative correlation (r^2 = 0.16). Finally, Fig. 1 (lower right) shows a relatively high qualitative (330/373; 88%) and quantitative (r^2 = 0.44).concordance between the the BBA and EIA results.

Comparison of serostatus interpretations

Table 2 shows the comparison between IFA and consensus interpretations of the stage of infection. There were 286/387 (73.9%) concordant interpretations and 74/387 (19.1%) discordant ones, whereas 27/387 (7.0%) samples had no consensus by the majority rule. This relatively low concordance was mostly the result of infection classified as indeterminate by IFA with the anti-VCA IgG+ and anti-EBNA IgG− serostatus pattern and as past infection by the consensus (due to EIA and BBA positive anti-EBNA IgG results). This was the case for 56 serum samples.

Table 3 shows the comparison between EIA interpretations and the consensus interpretations. There was a better concordance (329/387; 85%) with only 18/387 (4.7%) discordant interpretations. For 40 samples (10.3%), no comparison of interpretations could be made because consensus could not be reached or EIA serostatus was indeterminate (grey zone result). The relatively low sensitivity of EIA and BBA for anti-VCA IgM resulted in a reduced number of acute infection interpretations (82, 67 and 72 cases with IFA, EIA and BBA, respectively).

TABLE 2. Comparison of immunofluorescence assay and consensus interpretations

	Immunofluorescence assay				
Consensus	**Non-infected**	**Acute infection**	**Past infection**	**Indeterminate**	**Total**
Non-infected	85	–	–	–	85
Acute infection	–	82	–	3	85
Past infection	5	–	96	56	157
Indeterminate	3	2	3	23	31
Non plausible	2	–	–	–	2
No consensus	5	10	1	11	27
Total	100	94	100	93	387

TABLE 3. Comparison of enzyme immunoassay and consensus interpretations

	Enzyme immunoassay						
Consensus	**Non-infected**	**Acute infection**	**Past infection**	**Indeterminate**	**Non plausible**	**Grey zone**	**Total**
Non-infected	80	–	–	3	–	2	85
Acute infection	–	67	–	9	–	9	85
Past infection	–	–	154	2	–	1	157
Indeterminate	–	–	3	27	–	1	31
Non plausible	–	–	1	–	1	–	2
No consensus	–	–	6	3	–	18	27
Total	80	67	164	44	1	31	387

TABLE 4. Comparison of bead-based assay and consensus interpretations

	Bead-based assay							
Consensus	**Non-infected**	**Acute infection**	**Past infection**	**Indeterminate**	**Non plausible**	**Grey zone**	**NSC**	**Total**
Non-infected	67	3	1	6	2	1	5	85
Acute infection	–	72	–	4	–	–	9	85
Past infection	–	–	135	2	2	–	18	157
Indeterminate	–	1	5	17	–	3	5	31
Non plausible	–	–	2	–	–	–	–	2
No consensus	–	1	7	7	–	2	10	27
Total	67	77	150	36	4	6	47	387

NSC, nonspecific coating.

Table 4 shows the comparison of consensus interpretations of the BBA. There were 291/387 (75.2%) concordant interpretations but only 28/387 (7.2%) discordant ones. For 64 samples (17.6%), no comparison could be made because consensus could not be reached, or because of invalid NSC results in the BBA.

Because there was a substantial discrepancy between IFA and the other two methods for the anti-EBNA IgG results, we reviewed the clinical charts of the 70 patients with the anti-VCA IgG+, IgM–, anti-EBNA IgG– serostatus pattern and found that only 28 of these patients had evidence of immunosuppressive conditions or treatment that may explain a negative anti-EBNA IgG result in a patient with past infection. Another cause of discrepancy may be young age. The 42 patients without immunosuppressive conditions were aged from 1 month to 87 years (median 29 years) with a 25th percentile at 19 years. We also reviewed the BBA anti-EA IgG results of these 70 patients). Fifty were anti-EA IgG-negative, ten were positive, seven were in the grey zone and three were invalid (two due to NSC), without any obvious relationship with the patient's immune status.

IFA is acknowledged as the reference method in EBV serology. We therefore compared the IFA results with those of the newer methods (EIA and BBA) (Tables 5 and 6). The major difference compared with Tables 3 and 4 is in the classification of past infections (as defined by EIA and BBA) as indeterminate infections by IFA, again indicating a relatively low sensitivity of our IFA for anti-EBNA IgG.

Discussion

In the present study, we compared the diagnostic performance of a recently developed multiplexed BBA that allows the determination of antibody responses to several antigens in the same reaction. In the absence of a gold standard to ascertain the status of EBV infection, the diagnostic performance of this assay was assessed by comparison with the

TABLE 5. Comparison of enzyme and immunofluorescence assay interpretations

	Enzyme immunoassay						
Immunofluorescence assay	**Non-infected**	**Acute infection**	**Past infection**	**Indeterminate**	**Non plausible**	**Grey zone**	**Total**
Non-infected	80	–	6	6	1	7	100
Acute infection	–	65	2	12	–	15	94
Past infection	–	–	93	5	–	2	100
Indeterminate	–	2	63	21	–	7	93
Total	80	67	164	44	1	31	387

TABLE 6. Comparison of bead-based and immunofluorescence assay interpretations

	Bead-based assay							
Immunofluorescence assay	**Non-infected**	**Acute infection**	**Past infection**	**Indeterminate**	**Non plausible**	**Grey zone**	**NSC**	**Total**
Non-infected	67	3	12	7	2	2	7	100
Acute infection	–	69	–	10	–	1	14	94
Past infection	–	–	81	6	1	–	12	100
Indeterminate	–	5	57	13	1	3	14	93
Total	67	77	150	36	4	6	47	387

NSC, nonspecific coating.

reference methods and with a consensus of the results for all three methods determined by a majority rule. Compared with previous studies assessing the diagnostic performance of similar assays, the present study had more samples that were representative of each serostatus pattern determined by IFA [5–7]. It also had immunofluorescence data available for all samples, and not just for EIA/BBA discordant results as was the case in the study by Binnicker *et al.* [8].

Overall, we observed a good qualitative correlation between the three methods for detecting anti-VCA IgG and IgM antibodies. Of note, the serum samples had been frozen and stored for several years before testing by EIA and BBA, although we did not observe a reduced sensitivity for these tests compared with IFA that had been performed on fresh unfrozen sera. Thus, discrepancies cannot be attributed to antibody loss as a result of storage. Another potential source for discrepancies could be the use of different antigens in these tests. Both EIA and BBA used recombinant gp125 VCA, whereas the IFA assay used HR1 cells that express this antigen and other lytic antigens [9].

Compared with IFA and EIA, BBA had a slightly reduced sensitivity for the detection of anti-VCA IgM, resulting in in less frequent interpretations of acute infection.

By contrast, we observed a clearly reduced sensitivity of IFA compared to the two other methods for the detection of anti-EBNA antibodies. Although the two latter tests use recombinant EBNA-1 as antigens, IFA was performed using (in accordance with the manufacturer's instructions) 'lymphoid cells chosen for their selective production of EBNA antigens'. We could not obtain more details, although it is likely that these are latently EBV-infected cells that may express more latent antigens than just EBNA-1. If anything, this should result in IFA being more sensitive than the other assays. Second, anti-EBNA-1 IgG antibodies have been decribed as appearing later than other anti-EBNA antibodies but with life long persistence [3]. Thus, if the cells used for IFA testing expressed little EBNA-1, this would account for the apparent lack of sensitivity of IFA in patients with a pattern otherwise reflecting past infection (anti-VCA IgG+, anti-VCA IgM−).

Anti-EBNA IgG antibodies have been reported to disappear during EBV reactivation in immunocompromised patients [3,10]. However, among patients with discordant anti-EBNA IgG results (in the setting of positive anti-VCA IgG and negative IgM), only a minority had a history of immunosuppressive conditions, and even less detectable anti-EA antibodies that are a marker for EBV reactivation [3]. Therefore, it is likely that, in the majority of discordant results where anti-EBNA antibodies were detected by EIA and/or BBA, these were true positives in patients with past infection.

Indeterminate (type I) results might also occasionally occur in acute infection in the absence, or after rapid disappearance, of anti-VCA IgM in paediatric patients. However, only a minority of our patients were children. We also included type II indeterminate samples (anti-VCA IgM+, anti-VCA IgG+ and anti-EBNA IgG+) in our study. In both types of indeterminate results, the assessment of anti-VCA IgG avidity may be helpful in deciding the stage of infection [3]. Finally, we cannot exclude the possibility that these discrepancies are consistant false positive anti-EBNA IgG results with EIA and BBA as a result of long-term freezing.

Titres of anti-VCA IgG assessed by EIA have been reported to carry useful information as markers for EBV reactivation and correlate with EBV DNA load in the blood in various EBV-related malignancies, such as Hodgkin's lymphoma [11] or nasopharyngeal carcinoma [12]. It is important in this respect to note the poor quantitative correlation between BBA and EIA indices and immunofluorescence titres. This suggests that anti-VCA IgG results should not be interpreted quantitatively, irrespective of the method used. Although our quantitative discrepancies between BBA and IFA or EIA and IFA may be ascribed to differences in antigens, it is more difficult to explain the discrepancy between BBA and EIA because both are based on the same antigen. This lack of correlation may in part be the result of diluted samples not being run when samples gave out of range signals, which occurred in a substantial number of samples, as can be seen by the funnelling of dots in the upper right corner of the graph in Fig. 1 (BBA vs. EIA anti-VCA IgG; lower central graph).

In any case, the diagnosis of nasopharyngeal carcinoma should include testing for primarily IgA directed against various EBV antigens by IFA and BBA [13].

The concordance of BBA appeared lower than the concordance of EIA with the consensus, although this was related to the implementation of an internal control in the BBA that detected NSC in samples that may otherwise give a false positive result. At the time of the study, the cut-off values for NSC were relatively low, leading to the designation of 58 results as invalid, of which 47 were the result of NSC. Forty-four of those sera were available for retesting with a new version of the kit (reset threshold and new wash buffer). Of those, none tested NSC for IgG and 12 of 44 remained NSC for IgM. In addition, this residual problem could be solved by the use of an IgG absorption step before IgM testing as demonstrated in prospective samples.

In conclusion, BBA compared favourably with the reference IFA and produced similar resuts to the EIA methods. Both BBA and EIA offer a substantial saving in time as well as sample size (10 and 20 μL, respectively, vs. 60 μL for IFA)

and do not require training in fluorescence microscopy. However, BBA requires only two assays compared to one assay per antibody specificity for EIA and IFA. Finally, the BBA requires a costly flow cytometry reader.

Author contributions

PM designed the study, and participated in the data analysis and writing of the manuscript. OD performed the data reduction, and participated in the data analysis and writing of the manuscript, in the completion of a MD degree.

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Transparency Declaration

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