Evaluation of three different assays for the assessment of Epstein Barr Virus immunological status

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SUMMARY

Various attempts have been made to improve Epstein Barr Virus serodiagnosis by developing convenient methods. The present study evaluated the performance of multiplexed bead assays and immunoblot based assays on automated platforms by comparing them with immunofluorescence based assays for the determination of EBV immune status. A total of 45 serum samples were included in the study. Serum samples were tested by multiplexed bead EBV assays (AtheNA Multi-Lyte, Zeus Scientific,USA) and immunoblot based assays (Euroline, Euroimmun AG, Germany) on automated platforms. Assay systems were evaluated by comparing them with immunofluorescence based assays (Zeus Scientific, USA).

For EBV anti-VCA IgM, anti-VCA IgG, anti-EA and anti-EBNA, the kappa values reflecting agreements of AtheNA and IFA were 0.20, 0.54, 0.92 and 0.95 for anti-EA, anti-VCA IgG, anti-VCA IgM and anti-EBNA respectively and the agreements of Euroline and IFA were 0.53, 0.67, 0.81 and 1.000 for anti-VCA IgG, anti-EA, anti-VCA IgM and anti-EBNA respectively.

The results of the study performed on a limited number of serum samples demonstrated that the multiplexed bead assays and immunoblot assays agree with the standard IFA assay for anti-EBNA IgG and anti-VCA IgM detection while the agreement is less for anti-EA and anti-VCA IgG.

KEY WORDS: Epstein Barr Virus, Immunoblot based assay, Indirect immunofluorescence assay, Multiplexed microparticle-based assay

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INTRODUCTION

Epstein-Barr virus (EBV), a member of the family of human herpesviruses, infects up to 95% of adults worldwide. The virus produces diseases that range from acute self-limited infection to malignant neoplasms. EBV is implicated in diseases such as infectious mononucleosis, Burkitt's lymphoma, and nasopharyngeal carcinoma. Most EBV infections occur during childhood and are asymptomatic. When EBV infections are symptomatic, however, they may resemble other viral infections and require laboratory tests to confirm the presence of EBV (Ooka T. *et al.,* 1991; Linde A., 2003).

The presence of atypical lymphocytes on a peripheral smear supports the diagnosis of infectious mononucleosis but these are not universally detectable. The detection of heterophile antibodies and specific antibodies against EBV antigens has proven useful in the diagnosis of acute EBV infection (Linde A., 2003; Paul J.R., Bunnell W.W., 1932; Paul J.R. *et al.*, 2004). The diagnosis of a primary EBV infection builds upon an EBV-specific test for immunoglobulin G (IgG) and IgM antibodies to viral capsid antigens (VCA) and IgG

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antibodies to the EBV nuclear antigens (EBNA), especially EBNA-1, as the minimal requirement (Middletorp J.M. *et al.,* 1988). The humoral response to EBV infection is commonly evaluated using indirect immunofluorescence assay (IFA), or enzyme immunoassay (EIA) formats. IFA assays to date serve as the gold standard of EBV serodiagnosis (Hess R.D., 2004). However, these methods are time-consuming and are not suitable for large-scale testing or automatic handling. Furthermore, because of the variability of antigen-producing cells as well as subjective reading of the results, they are difficult to standardize. Various attempts have been made to improve EBV serodiagnosis by developing more convenient methods (Farber I., 2001).

In the present study, the performance of multiplexed microparticle-based EBV assays (EBV VCA IgM Test System and EBV IgG Test System AtheNA Multi-Lyte, Zeus Scientific, USA) on the Athena Multi-Lyte System instrument (Luminex 100IS, Zeus Scientific, USA,) and immunoblot based assays performed on Profiblot instrument (Tecan, Switzerland) were evaluated by comparing them with immunofluorescence based assays for the detection of anti-VCA IgG and IgM, anti-EBNA IgG, and anti-Early Antigen (anti-EA) IgG antibodies. In addition, the agreement between the three different systems in categorizing patients' EBV status was assessed.

MATERIALS AND METHODS

Patient samples

A total of 45 leftover serum samples from patients aged one to 62 years (mean age 25.4) sent to the Ege University Hospital, Department of Clinical Microbiology Laboratory for EBV testing were included in the study.

EBV testing

Athena Multi-Lyte (Zeus Scientific,USA) EBV IgG and EBV anti-VCA IgM analyses were performed as described in the manufacturers' package inserts on the AtheNA Multi-Lyte System instrument (Luminex 100-IS, Zeus, USA) automated platform. Athena Multi-Lyte EBV IgG Test system is intended for the qualitative detection of IgG class antibody to three separate EBV Antigens (EBV-VCA gp-125, total EBV-EA and recombinant EBNA-1) in human serum. Briefly, dyed (fluorescent) bead sets, each of which is coated with a different, specific EBV antigen, are mixed with diluted patient sample. The bead mix also contains one bead set designed to detect non-specific binding and four separate bead sets used for assay calibration. After incubation and a wash cycle, an anti-human IgG or IgM antibody conjugated with phycoerythrin (PE) is added. The instrument can then identify beads based on the fluorescence of the dyes and quantify antibody on each bead based on the fluorescence of the PE. This system can assess the response to multiple independent antigens in a single incubation. Same samples were also tested with Euroline IgM and IgG test kits (Euroimmun, Germany) using the Profiblot instrument (Tecan, Switzerland). Euroline test kit provides a qualitative assay for human antibodies of the IgG class to five different EBV antigens: VCA gp125, VCA p19, EBNA 1, p22 and EA-D. The test kit contains test strips coated with parallel lines of antigens. In the case of positive samples, the specific IgG or IgM antibodies will bind to the corresponding antigenic site. To detect the bound antibodies, a second incubation is carried out using an enzyme-labelled anti-human IgG capable of promoting a color reaction. Samples were also tested by EBV VCA IgM , VCA IgG, EA and EBNA IFA test system (Zeus Scientific, USA).

Interpretation of assay results for categorizing patients

The interpretation criteria used conformed to the manufacturers' guidelines for the assays. There are some differences in interpretation criteria of the test systems for categorizing reactivation, past infection and indeterminate patterns while interpretation criteria of seronegative and primary infections are identical.

Statistical Analysis

Kappa statistics in SPSS 13.0 statistical package programme was used for data analysis and to measure agreement between the tests. To interpret the degree of agreement, the guidelines provided by Landis and Koch were used: kappa values ≤0.40 poor to fair agreement, 0.41-0.60 moderate agreement, 0.61-0.80 substantial agreement, 0.81-1 almost perfect agreement (Landis J.R. *et al.,* 1977).

RESULTS

The Athena Multi-Lyte system and Euroline test systems were compared with IFA as the reference method. As seen in Table 1, Kappa values of Athena and IFA were 0.20, 0.54, 0.92 and 0.95 for anti-EA, Anti-VCA IgG, anti-VCA IgM and anti-EBNA respectively. While there is a strong agreement between these two systems for the anti-VCA IgM and anti-EBNA assays, the agreements for anti-VCA IgG assay was 'moderate' and for anti-EA IgG assay 'poor'.

As seen in Table 2, the kappa values of Euroline and IFA were 0.53, 0.67, 0.81 and 1.000 for Anti-VCA IgG, anti-EA, anti-VCA IgM and anti-EBNA respectively. There was strong agreement between the two systems for the anti EBNA and for anti-VCA IgM, 'substantial agreement' for anti-VCA IgG assay and 'moderate agreement' for anti-EA. EBV infection status of the patients categorized

TABLE 1 - *Agreement of anti-EBV serology between Athena and IFA.*

Result of assay with the AtheNA	*No of sera that gave IFA result that was:*			*Kappa value*
	Positive	*Negative*	*Equivocal*	
Anti-VCA IgG				
Positive	32	2	-	0.54
Negative	5	6		
Anti-VCA IgM				
Positive	7	1	-	0.92
Negative	0	35	2	
Anti-EBNA				
Positive	28	1	-	0.95
Negative	-	16	-	
Anti-EA				
Positive	3	5	-	0.20
Negative	6	31	-	

TABLE 2 - *Agreement of anti-EBV serology between Euroline and IFA.*

Result of assay with the Euroline	*No of sera that gave IFA result that was:*			*Kappa value*
	Positive	*Negative*	*Equivocal*	
Anti-VCA IgG				
Positive	28	0	-	0.53
Negative	9	8	-	
Anti-VCA IgM				
Positive	5	0	0	0.81[a]
Negative	2	36	2	
Anti-EBNA				
Positive	28	0		1.000
Negative	0	17	-	
Anti-EA				
Positive	7	3	-	0.67
Negative	2	33	-	

[a]Two equivocal IFA results were excluded for kappa analysis.

TABLE 3 - *Comparison of serological pattern interpretations in determining EBV infection status between Euroline, Athena and IFA[a].*

Diagnosis	*Number of samples with the given EBV infection status*		
	AtheNA (%)	*Euroline (%)*	*IFA (%)*
EBV seronegative	7 (% 15.56)	10 (% 22.22)	8 (% 17.78)
Primary infection	7 (% 15.56)	5 (% 11.11)	6 (% 13.33)
Past infection or reactivation	24 (53.33)	25 (% 55.56)	26 (57.78)
Unresolved	7 (% 15.56)	5 (% 11.11)	5 (% 11.11)
Agreement with IFA (Kappa)	0.57	0.67	-

[a]Interpretation of VCA IgG, VCA IgM, VCA-EA, EBNA-1 IgG results in accordance with the manufacturer's interpretation criteria.

as seronegative, primary infection, past infection or reactivation and unresolved cases were interpreted according to the serological patterns of the patients, in accordance with manufacturer's interpretation criteria for each test system. The agreement between the two systems compared to IFA in categorizing patients' EBV status were 0.57 (moderate) for AtheNA and 0.67 (substantial) for Euroline (Table 3).

DISCUSSION

Several assays are available and are routinely used for the diagnosis of EBV infection that employ various techniques but differ greatly in performance (Buisson M. *et al.,* 1999; Debyser Z. *et al.,* 1997; Gartner B.C. *et al.,* 2001; Svahn A. *et al.,* 1997; Weber B. *et al.,* 1996). The present study was conducted to compare multiplex bead assay and immunoblot assay with IFA.

The conventional methods used to perform EBV testing are based on EIA and IFA. Although these methods are well characterized and reliable, they require an aliquot of sample and reagents for each parameter tested. In addition, the turnaround time and the cost of labour and materials rises proportionally with each test performed. In order to circumvent these issues, the idea of multiplexing EBV testing was developed (Baetens D.G.A., 2001).

In a previous study evaluating a multiplexed bead assay, the concordance between the assay and conventional IFA with regard to categorizing the EBV immune status was found to be 82% (Paul J.R. *et al.,* 2004). In our study, the kappa value was calculated to evaluate the agreement between the bead system and IFA. To interpret the degree of agreement, although the divisions are arbitrary, the guidelines provided by Landis and Koch were used (Landis J.R. *et al.,* 1977). The agreements were strong for anti-VCA IgM and Anti-EBNA. On the other hand, the agreement for anti VCA IgG assays was moderate and for Anti-EA IgG assays poor. Moderate agreement (kappa value 0.57) was found in assessment of patients' EBV immunologic status.

Blot techniques are considered highly specific and mostly used as confirmatory methods (Hess R.D., 2004). One study proposed immunoblot techniques with recombinant antigens as the new gold standard in EBV serology (Bauer G., 2001). Automated blotting and scanning systems provide a convenient alternative in routine EBV testing. In our study, the agreement of immunoblot test and IFA was very good for anti- EBNA and anti-VCA IgM, substantial for Anti-EA and moderate for anti-VCA IgG. The agreement with IFA was slightly better (kappa value is 0.67 categorized as substantial agreement) than the one obtained with the bead assay in the assessment of patients' immunologic status.

The most common use of EBV serology is to help distinguish acute EBV disease from nonacute forms. In our study, the number of cases with acute infection was limited. IFA detected six patients with acute infection, Euroline could not detect one of them and one more acute infection

was diagnosed with AtheNA system. When the patients with discordant results for acute infection were analysed, it was noted that the patient categorized as having acute infection with AtheNA, had VCA IGM reactivity which is close to the cut-off value. This sample was obtained from a liver transplant donor without any symptoms. By IFA, the patient was accepted as indeterminate since anti-VCA IgG and anti-EA were positive. On the other hand, this profile may reflect the late phase in acute infection. With the Euroline system, only anti-VCA IgG and p22 antibodies were positive (considered as past infection according to the instructions). Since Euroline and IFA gave atypical profiles for this patient, it was not possible to reach a definite conclusion in diagnosing the stage of the disease. The other sample positive both in Athena system and IFA was seronegative by Euroline assay which may indicate that Euroline is falsely negative. This was a child admitted to the hospital with fever and acute respiratory infection symptoms without lymphadenopathy.

The different performances of the individual parameters can be explained by the various antigens used. There are many antigen preparations used in different kits, ranging from cell extracts to recombinant proteins to synthetic peptides (Linde A., 2003). The Euroline test strips were coated with native VCA gp 125 purified by affinity chromatography, recombinant VCA p19 antigen, recombinant EBNA-1 antigen, recombinant p22 capsid antigen and recombinant EA-D antigen. In AtheNA Multi-Lyte System, polystyrene beads are conjugated with affinity purified EBV VCA gp 125, affinity purified EA (roughly equal parts EA-D and EA-R) and recombinant EBNA-1. In the EBV-VCA IFA test system, slides contain infected cells in each well.

The preparation of antigens, the selection of antigens and substrates, the different techniques used, and even the interpretation of the results vary remarkably among different manufacturers. It is clear that standardization of serological assays for EBV infection is needed. IFA may still be considered the reference system for the serodiagnosis of EBV infection but it is laborious, not suitable for automatic handling, requires experienced personnel to read the images, time-consuming and equivocal results could still be obtained.

Multiplexed bead assay and immunoblot test system have a number of potential advantages over conventional microtiter plate serologic assays and IFA, including an automated platform. Furthermore, common test groupings, such as the assays performed to evaluate EBV serologic status can be performed in one tube or strip. These strategies can potentially lower cost, minimize aliquot errors, and reduce turnaround time and sample requirements. (Paul J.R., 2004).

The results of this study performed on a limited number of serum samples demonstrated that the multiplexed bead assays and immunoblot assays agree with the standard IFA assay for EBNA-1 IgG and anti-VCA IgM detection while the agreement is less for the other two parameters, anti-EA and VCA IgG.

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