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Comparison of Methods Used for the Diagnosis of Epstein-Barr Virus Infections in Children

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Abstract

The accurate diagnosis of Epstein-Barr virus (EBV) infections is important, as many other infectious agents or diseases can cause similar symptoms. In this study, sera of pediatric patients who were suspected to have an EBV infection, were sent to Eskisehir Osmangazi University Faculty of Medicine, Department of Clinical Microbiology, and investigated by IFA, ELISA, immunoblotting and Real-time PCR. The performances of these tests were compared with IFA. The rates of agreement between ELISA and IFA were found as 100% for seronegative, 100% for acute primary infection, 22.2% for late primary infection, 92.1% for past infection. The rates of agreement between immunoblotting and IFA were found as 80.8% for seronegative, 68.8% for acute primary infection, 55.6% for late primary infection, 86.6% for past infection. The sensitivity of immunoblotting for anti-VCA IgM was identical with ELISA, and higher for anti-VCA IgG, anti-EBNA IgG, anti-EA antibodies, while the specificity of immunoblotting for these antibodies were found to be lower. The sensitivity and specificity of Real-time PCR for detection of viremia in acute primary infection were found as 56.25% (9/16) and 97.89% (139/142), respectively. The diagnostic methods should be chosen by evaluating the demographic characteristics of patients and laboratory conditions together.

Key words: ELISA, Epstein-Barr virus, immunoblotting, Indirect Fluorescence Assay (IFA), Real-time PCR

Introduction

EBV was discovered in 1964 by electron microscopy of suspension cultures of African Burkitt lymphoma cells (Epstein *et al.,* 1964). EBV is a member of Herpesviridiae family and is a double stranded DNA virus (Martinez and de Gruijl, 2008). It is known as human herpesvirus 4 (Odumade *et al.,* 2011).

EBV is an important agent that affects nearly all adults throughout the world (Kreuzer *et al.,* 2013). In Turkey, seropositivity rates in adult age are found between 70–99.4% (Zeytinoğlu *et al.,* 2005; Ozkan *et al.,* 2003). Primary EBV infection is usually seen as asymptomatic infection in childhood. Generally, younger children are moderately ill, and the severity of primary EBV infection in adult increases with age (Odumade *et al.,* 2011). During primary infection, EBV can cause a wide variety of symptoms, depending on the host age and immune status, ranging from asymptomatic infection to severe infectious mononucleosis (IM) with complications (Ebell, 2004; Gärtner *et al.,* 2003). IM typically begins with malaise, and followed by fever, sore throat, swollen cervical lymph nodes and fatigue. Some patients experience an abrupt influenza-like onset, with fever, chills, body aches and sore throat (Odumade *et al.,* 2011). Cytomegalovirus (CMV), Rubella virus, *Toxoplasma gondii* infections and hematologic malignancies can cause similar symptoms. Therefore differential diagnosis of these agents is important.

EBV is intermittently shed from saliva. The main route of transmission is orally from person to person although transmission *via* blood products, transplantation, and sexual transmission were shown (Schooley, 1995; Woodman *et al*., 2005). Being commonly transmitted through saliva; acute IM is called "the kissing disease" (Thompson, 2015). Virus can affect B and T lymphocytes, epithelial cells and smooth muscle cells, and can cause malign transformation. It is associated with

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Burkitt lymphoma, nasopharyngeal carcinoma, post-transplantation lymphoproliferative disease, Hodgkin and non-Hodgkin lymphoma, gastric carcinoma and leiomyosarcoma (Duca *et al.,* 2007).

In immunocompetent individuals, EBV infection is controlled by the humoral and cellular immune responses, in cooperation with the interferon system. EBV reactivation in immunocompetent individuals may not cause any specific symptoms. But in immunocompromised individuals such as solid organ or bone marrow recipients, and HIV infection, virus reactivation can cause serious complications (Jacobson and LaCasce, 2010).

Formerly, for the diagnosis of acute primary EBV infections, peripheral blood smear (atypical lymphocytes-Downey cells), monospot and Paul-Bunnell agglutination test could be used, but as these tests are not specific to the EBV infection and false negativity rates are high in children aged under 4 especially in Paul-Bunnell test (Horwitz *et al.*, 1981), evaluating specific antibodies by Immunofluorescence Assay (IFA), Enzyme Linked Immuno Sorbent Assay (ELISA), Immunoblotting and searching for DNA by molecular methods are used recently. In immunocompromised individuals, evaluating viral DNA by molecular methods should be used because of the inefficient antibody response.

In this study, serum samples of pediatric patients who were suspected to have an EBV infection were sent to Eskisehir Osmangazi University Medicine of Faculty, Department of Medical Microbiology, and investigated by IFA, ELISA, Immunoblot and Real-time PCR. The performances of these tests were compared with the gold standard test IFA and their sensitivities and specificities were evaluated.

Experimental

Material and Methods

Design of study. One hundred and seventy-eight consecutive samples that were received between February-June 2013 for EBV serology, from pediatric patients (median age 72 months; range: 24–216 months; 56.7% male, 43.3% female) applied to Eskisehir Osmangazi University Medical Faculty with lymphadenopathy, pharyngitis, fever, hepatomegaly and/or splenomegaly were included in this study. For the control group, 30 healthy pediatric individuals (median age 78 months; range: 24–204 months; 43.3% male, 56.7% female) were included. In a total of 208 serum samples, EBV specific antibodies were studied by IFA, ELISA, immunoblotting, and EBV DNA was evaluated by Real-time PCR.

Methods. EBV specific antibodies were evaluated by indirect IFA, ELISA and immunoblotting in serum samples, and results were interpreted according to the manufacturers. Viremia was evaluated by real-time PCR in all patients except seronegatives.

IFA. EBV anti-viral capsid antigen (VCA) IgG, anti-VCA IgM, anti-early antigen (anti-EA) IgG, anti-Epstein-Barr nuclear antigen (EBNA) IgG and anti-VCA IgG avidity tests were evaluated simultaneously with commercial IFA kit (Euroimmun AG, Lüebeck, Germany). The wells of the slides were prepared from human EBV transformed cell lines obtained from Burkitt lymphoma patients and the cell lines P3HR1 expressing VCA, Raji cell expressing EBNA complex, and EU-33 expressing EA antigen. The test was studied according to the manufacturer's instructions and the slides were evaluated under × 20 and × 40 objectives of fluorescent microscope in accordance with the manufacturer's instructions.

ELISA. Anti-VCA IgG, anti-VCA IgM, anti-EBNA IgG, anti-EA IgG antibodies and anti-VCA IgG avidity were evaluated by Euroimmun AG, Lüebeck, Germany ELISA kits according to the recommendations of manufacturer. For anti-VCA IgG avidity, serum samples were studied duplicated one with urea (8 M concentration) and one without. At the end of the study, relative avidity index was calculated, and values up to 40 were considered as low avidity (indicating recently acquired infection) and those exceeding 60 were considered as indicative of high avidity. Values between 40 and 60 were evaluated as grey zone.

Immunoblotting. Euroline anti-EBV profil 2 Immunoblot kit (Euroimmun, Germany) was used to detect EBV specific anti-VCA IgM, anti-VCA IgG, anti-EBNA-1 IgM, anti-EBNA-1 IgG, anti-p22 IgM, anti-p22 IgG, anti-EA IgM and anti-EA IgG antibodies. This kit contains test strips coated with parallel lines of different antigens. In the case of positivity, the specific IgG or IgM antibodies bind to the corresponding antigenic site, and then to detect the antibodies, another incubation is performed using an enzyme-labelled anti-human Ig to create a color change. Test was performed and at the end of the study the strips were scanned and evaluated by software according to the instructions of manufacturer.

Real-time PCR. EBV Real-time PCR Kit, Exicycler™ 96 thermal block, ExiCycler 96 Real-time PCR (Bioneer, Korea) system were used to detect EBV DNA quantitatively in serum samples. DNA isolation was performed by ExiPrep viral DNA/RNA extraction kit and device (Bioneer, Korea). In PCR stage, lyophilized PCR mix was diluted and DNA was added. Then patient samples and five standards were loaded to the system. In PCR stage the cycle was as following: predenaturation at 95°C for 5 min., 45 cycles of denaturation at 95°C for 5 s and annealing and detection at 55°C for 5 s. Data were evaluated by ExiDiagnosis (Diagnostic Soft. ver. 1.27.3).

Results

EBV infectious serology was evaluated in patient (n = 178) and control (n = 30) groups by ELISA and immunoblotting tests, and results were compared with IFA as the reference method. EBV DNA was searched in sera by real-time PCR to evaluate viremia.

EBV infection stages were determined by IFA reference method, according to the EBV diagnosis standards (Linde and Falk, 2007). In patient group, 26 (14.6%) seronegativity, 16 (9.0%) acute primary infection, 9 (5.1%) late primary infection, 127 (71.3%) past infection were determined by IFA. In control group, 25 (83.4%) past infection, 4 (13.3%) seronegativity and 1 (3.3%) late primary infection stages were determined.

EBV infection stages were also determined by ELISA and immunoblotting methods, and these results were compared with IFA results (Table I and Table II).

In patient group, ELISA and IFA concordance was as 100% for seronegativity, 100% for acute primary infection, 22.2% for late primary infection, 92.1% for past infection. Immunoblotting and IFA concordance was as 80.8% for seronegativity, 68.8% for acute primary infection, 55.6% for late primary infection, 86.6% for past infection. In control group, the concordance of ELISA and immunoblotting with IFA were as 100%.

When considering specific antibodies, the results obtained by ELISA and immunoblotting were compared with IFA results, and the sensitivities, specificities, positive predictive values and negative predictive values of methods were evaluated (Table III and IV).

The sensitivity of immunoblotting method was found similar with ELISA method for anti-VCA IgM, and was higher than ELISA for the other three antibodies, but the specificity was lower than ELISA for all four antibodies. Table V and Table VI show the PCR results and viral loads according to the infection stages, respectively. DNA was not detected in the control group. The sensitivity of real-time PCR in detecting viremia during acute primary infection is 56.25% (9/16), and the specificity is 97.89% (139/142).

Table I
Comparison of stages determined by ELISA and IFA in patient group.

ELISA serological stage	IFA serological stage				
	SN	API	LPI	Past	Total
SN	26	–	–	2	28
API	–	16	5	1	22
LPI	–	–	2	7	9
Past	–	–	2	117	119
Total	26	16	9	127	178

SN = seronegative, API = acute primary infection, LPI = late primary infection

Table II
Comparison of stages determined by immunoblotting and IFA in patient group.

ELISA serological stage	IFA serological stage				
	SN	API	LPI	Past	Total
SN	21	–	–	1	22
API	–	11	2	2	15
LPI	2	5	5	14	26
Past	3	–	2	110	115
Total	26	16	9	127	178

IB = immunoblotting, SN = seronegative, API = acute primary infection, LPI = late primary infection

Discussion

In immunocompetent individuals, the main aim to evaluate serological antibodies is to diagnose acute primary infection (Gärtner *et al.*, 2003). Three antibodies are essential for the detection of infection stage in

Table III
Comparison of antibody results obtained by ELISA with IFA results in patient group.

ELISA		IFA		ELISA				
		Pos.	Neg.	Sens.	Spec.	PPV	NPV	Kappa value
Anti-VCA IgM	Pos.	20	7	83.33	95.45	74.07	97.35	0.748 (Substantial agreement)
	Neg.	4	147					
Anti-VCA IgG	Pos.	145	1	96.03	96.30	99.32	81.25	0.858 (Almost perfect agreement)
	Neg.	6	26					
Anti-EBNA IgG	Pos.	114	5	91.20	90.57	95.80	81.36	0.792 (Substantial agreement)
	Neg.	11	48					
Anti-EA IgG	Pos.	6	2	26.09	98.71	75	90	0.343 (Fair agreement)
	Neg.	17	153					

Pos. = positive, Neg. = negative, sens. = sensitivity, spec. = specificity, PPV = positive predictive value, NPV = negative predictive value

Table IV
Comparison of antibody results obtained by immunoblotting with IFA results in patient group.

IB		IFA		IMMUNOBLOTTING				
		Pos.	Neg.	Sens.	Spec.	PPV	NPV	Kappa value
Anti-VCA IgM	Pos.	20	15	83.33	90.26	57.14	97.20	0,617 (Substantial agreement)
	Neg.	4	139					
Anti-VCA IgG	Pos.	150	6	99.34	77.78	96.15	95.45	0.835 (Almost perfect agreement)
	Neg.	1	21					
Anti-EBNA IgG	Pos.	120	15	96	71.70	88.89	88.37	0.716 (Substantial agreement)
	Neg.	5	38					
Anti-EA IgG	Pos.	17	25	73.91	83.87	40.48	95.59	0.427 (Moderate agreement)
	Neg.	6	130					

IB = immunoblotting, Pos. = positive, Neg. = negative, sens. = sensitivity, spec. = specificity, PPV = positive predictive value, NPV = negative predictive value

Table V
Evaluation of real-time PCR results based on infectious stages by IFA, ELISA and immunblotting methods in patient group.

		Real-Time PCR			Total
		Detected	Not detected	N/A	
IFA	Seronegative	–	6	20	26
	Acute primary infection	9	7	–	16
	Late primary infection	2	7	–	9
	Past infection	1	126	–	127
ELISA	Seronegative	–	8	20	28
	Acute primary infection	11	11	–	22
	Late primary infection	–	9	–	9
	Past infection	1	118	–	119
IB	Seronegative	–	2	20	22
	Acute primary infection	7	8	–	15
	Late primary infection	5	21	–	26
	Past infection	–	115	–	115
Total		12	146	20	178

IB = immunoblotting, N/A = not applied

immunocompetent patients: anti-VCA IgM, anti VCA IgG and anti-EBNA-1 IgG. Presence of anti-VCA IgM and anti-VCA IgG, absence of anti-EBNA IgG means acute primary infection, and presence of anti-VCA IgG and anti-EBNA with absence of anti-VCA IgM means past infection (De Paschale and Clerici, 2012). Isolated anti-VCA IgG positivity, isolated anti-EBNA positivity and the presence of all three parameters are difficult to interpret. In these situations, to evaluate IgM and IgG antibodies with immunoblotting, anti-VCA IgG avidity test, anti-EA/D antibodies and to investigate viral genome with molecular methods are recommended (De Paschale and Clerici, 2012). In many instances different methods can be simultaneously used to detect the correct infection stage.

In this study, we compared the results of different methods with gold standard IFA test. In our patient group, the ratio of acute primary infection stage was as 9.0%, and late primary infection consisted of 5.1% of patients. All of the acute primary infection patients (n:16) were also interpreted as acute primary infection by ELISA method. But six other patients were interpreted as acute primary infection by ELISA although five of them were late primary, and one was past infection by IFA. The compliance value of ELISA and IFA was as 100% for seronegativity and acute primary infection, and 92.1% for past infection, but this value was low for late primary infection. Only two of 9 late primary infections were interpreted correctly by ELISA. Five patients were interpreted as acute primary infection, and 2 were as past infection.

By ELISA, four specific antibodies are evaluated (anti VCA IgM, anti VCA IgG, anti EBNA IgG and anti EA IgG). When solely anti VCA IgG is positive, it is recom-

Table VI
Distribution of real-time PCR positive results on the basis of stages according to different methods.

Real-time PCR Viral load (copies/ml)	IFA Stage	ELISA Stage	Immuno-blotting Stage
92	Past	Past	Late primary
96.9	Acute	Acute	Late primary
283	Acute	Acute	Acute
1120	Acute	Acute	Acute
2470	Acute	Acute	Acute
5440	Acute	Acute	Late primary
6450	Acute	Acute	Late primary
6450	Late primary	Acute	Late primary
8550	Acute	Acute	Acute
8730	Late primary	Acute	Acute
10300	Acute	Acute	Acute
16700	Acute	Acute	Acute

mended to test anti VCA IgG avidity to distinguish late primary infection from past infection without anti EBNA. The avidity testing of VCA IgG may also help to resolve cases in which VCA IgM persists for a long term (Bauer, 2001). In our study, all of the patients with solely anti VCA IgG positivity (n = 9) had low avidity index values, therefore they were interpreted as late primary infection by ELISA. Two of them were interpreted as late primary infection, and seven were past infection by IFA. Four of these 9 patients were also solely positive for anti VCA IgG by IFA. They were interpreted according to their avidity results. In the literature, the presence of isolated anti VCA IgG profile occurs in 2–8% of immunocompetent patients tested for EBV (Klutts *et al.*, 2009; De Paschale *et al.*, 2009), and most of these cases are thought to correspond to past infections. In our patient group, isolated anti VCA IgG positivity was seen in 10 (5.6%) patients by standard method IFA. Two patients were diagnosed as late primary infection due to their avidity index values. Eight patients were interpreted as past infections. Seven of these eight patients were positive for anti-EBNA IgG, and negative for anti-EBNA IgM by immunoblotting, so they were interpreted as past infection. One of them had anti-EBNA IgM but not anti-EBNA IgG and was interpreted as late primary infection. Isolated anti-VCA IgG can be found in patients with past infection as non-appearance or loss of EBNA-1 IgG, and in patients with acute infection with the delayed appearance or early loss of anti-VCA IgM (De Paschale *et al.,* 2009). It is reported that anti-VCA IgM may appear 1–2 weeks after anti-VCA IgG or lasts for a short time, or anti-VCA IgM can be at low concentrations therefore it cannot be detected. Furthermore, some patients are negative for anti-EBNA IgG after recovery (De Paschale *et al.,* 2009; Bauer, 1995). In a study that evaluated EBV serological markers of 2422 patients with suspected EBV infection by ELISA, isolated anti-VCA IgG positivity rate is found as 7% (De Paschale *et al.,* 2009). In some instances, second sampling of blood is preferred to correctly diagnose the infection stage, but especially in children it may be difficult. It is important to correctly diagnose the stage in one sample. It may be possible by evaluating the anti-VCA IgG avidity simultaneously.

As mentioned above, all of the acute primary infection cases were diagnosed correctly by ELISA. Kreuzer *et al.,* (2013) compared two different ELISA assays with IFA method in 537 samples and reported the agreement values of assays as 100% for acute infection. The agreement values in seronegativity and past infection were as 89.7–100% and 98.1–99.1%, respectively. Schaade *et al.* (2001) compared the performances of EBV specific ELISA with indirect immunofluorescent reference method in 66 children patients and concluded that the ELISA system used were suitable for the diagnosis of seronegativity and acute EBV infection by that system could not determine recent primary and past infections correctly and had a high rate of indeterminate results. Also, they suggested that the ELISA system used was not applicable to the diagnosis of reactivated EBV infections. Devanthéry and Meylan (2010) evaluated EBV infection stages of 387 serum samples by three different methods and determined a consensus stage. They reported ELISA/consensus concordance as 85% (329/387). In 85 samples the consensus stage was acute infection, but ELISA could detect 67 of them correctly. In 18 samples the ELISA could give indeterminate or grey zone results. On the other hand, all of the acute infection results determined by ELISA were evaluated as acute infection by consensus. The sensitivity and specificity of ELISA kits are critical in stage evaluation. Especially the sensitivity of anti-VCA IgM is very important to detect acute infections. The EBV-specific tests differ in the substrates or antigens used, and the interpretation criteria are different among various manufacturers (Hess, 2004). The reference method IFA usually uses EBV-transformed B cell lines from Burkitt's lymphoma patients; on the contrary, most ELISA kits use purified native or recombinant antigens (Gärtner, *et al.,* 2003; De Paschale and Clerici, 2012). The most important thing in determining the correct stage is to choose high quality kits. ELISA is preferred in many laboratories as it is practical. As these assays can be automated, they can improve speed and efficiency of EBV testing, especially in high-volume laboratories.

When we compared immunoblotting stage results with IFA, we realized that the concordance rates were low. Five of 16 (31.2%) acute primary patients were evaluated as late primary infection. The ages of these

patients were 3, 3, 5, 10, and 12. Three of these patients were positive by PCR (96.9, 5440, 6450 copies/ml respectively) and all of these five patients were diagnosed as acute primary infection by ELISA. Immunoblotting method in our study uses recombinant antigens (VCA p19, EBNA-1, p22, EA-D) and one native purified antigen by chromatography (VCA gp125). Recombinant EBV specific antigens are accepted to be superior to lysate antigens, because they are not influenced by potential anti-cellular antibodies (Bauer, 2001; Gärtner *et al.,* 2001). Basically, infection stages can be evaluated according to anti-VCA IgM, anti-VCA IgG, anti-EBNA-1 IgG, anti-EA-D IgG and anti-p22 IgG. This method is highly specific and used mostly as a confirmatory method (Hess, 2004).

In our study, with the use of anti-VCA IgG avidity, we defined late primary infection stage by ELISA and IFA. To make accurate comparisons, we defined stages compatible with late primary infection by evaluating the antibodies detected by immunoblotting. As it is reported in the literature, during convalescence, anti-VCA IgM antibodies decrease, while anti VCA IgG antibodies rise and persist for life. Between the third and sixth months, anti-VCA IgM antibodies disappear but anti-EBNA-1 IgG antibodies become detectable. Therefore, anti-VCA IgM, anti-VCA IgG and anti-EBNA IgG may be present simultaneously in late primary infection and reactivation (Nystad and Myrmel, 2007). We also evaluated the anti-EBNA-1 IgM to be able to make correct comments on stages of infections. It is reported as an indicator of recent primary infection, and it is positive for 2–4 months after primary infection. Although there are a few commercially available anti-EBNA-IgM ELISA kits, anti-EBNA IgM is not used in routine diagnosis of EBV infections. In our study, we could be able to evaluate anti-EBNA IgM by immunoblotting IgM strips. 26 patients and 1 individual of the control group were evaluated as late primary infection. 20 of them were positive for anti-VCA IgM, anti-VCA IgG and anti-EBNA IgG, and in 10 anti-EBNA IgM was positive. In 7 samples, anti-VCA IgM and anti-EBNA IgG were negative but anti-VCA IgG was positive. According to their ages and/or anti-EBNA IgM results they were evaluated as late primary infection. By immunoblotting, some of the results were difficult to interpret. In routine diagnosis additional tests should be performed to diagnose the correct stage. But in our study, we aimed to evaluate the sufficiency of the tests alone. Although immunoblotting is suggested as a confirmatory test (Hess, 2004), we had some serious problems especially when anti VCA IgM, IgG and anti-EBNA IgG were simultaneously or when only anti VCA IgG was positive. Maybe we should report these results as "not determined" but we evaluated them as "late primary infection" and this may be our limitation.

We also aimed to measure the agreement of different methods in detecting the specific antibodies correctly as well as the correct staging, and evaluated sensitivity and specificity values. To interpret the degree of agreement, the guidelines reported by Cohen and Viera were used: Kappa value between 0.01–0.20 slight agreement, 0.21–0.40 fair agreement, 0.41–0.60 moderate agreement, 0.61–0.80 substantial agreement, 0.81–0.99 almost perfect agreement (Cohen, 1960; Viera and Garrett, 2005). According to these criteria, ELISA method had substantial or almost perfect agreement values for anti VCA IgM, IgG and anti-EBNA IgG. But this value was very low for anti-EA IgG. Therefore, we suggest that in immunocompetent patients, it is appropriate and sufficient to evaluate the infection stage by these three antibodies but not anti-EA IgG. Likewise, the agreement value of anti-EA IgG of immunoblotting method was low as moderate agreement. As immunoblotting is recommended as a confirmatory method, this low value is disappointing.

In the literature, anti-EA IgG is reported positive in approximately 85% of patients with acute infection, but in some cases, it can still be positive for years after acute primary infection. During reactivation or nasopharyngeal carcinoma, high titers of anti-EA IgG are seen (de Paschale and Clerici, 2012). It is also reported that, IgG reactivity against EA can be seen in all phases of infection, although it is predominantly seen during lytic infection (Nystad and Myrmel, 2007). Since an increase in the titer of anti-EA IgG can be considered as a marker of reactivation, it is suggested to evaluate anti-EA IgG only if serial sampling is possible (De Paschale and Clerici, 2012). In a study by Altuglu *et al.,* (2007), they reported the kappa value of anti-EA IgG of the same immunoblotting system as 0.67 (substantial agreement). But this value was as 0.20 for anti-EA IgG with automated bead assay. Klutts *et al.,* (2009) evaluated specific EBV antibodies and determined all possible phases that may occur. They reported that except for the relatively small number of primary acute infection patients, anti EA IgG was of little utility. Also Hess (2004) reported the positivity rate of anti-EA IgG in primary infection as 60–80% and suggested that in 20% of healthy individuals it is present. In this literature it is reported that EA specific serological parameters are not helpful to confirm any stage specific diagnosis.

In addition to the comparison of the methods in evaluating individual parameters, sensitivity and specificity values were also compared. The sensitivity of these two methods was the same for anti VCA IgM, and immunoblotting had slightly high values for anti VCA IgG and anti EBNA IgG. But the sensitivity performance of immunoblotting was significantly better for anti EA IgG. When we considered the specificity values, immunoblotting method had lower values. Immuno-

blotting assays are considered to have high specificity for EBV serology but unfortunately we had lower values than ELISA, especially for anti EBNA IgG (false positive results; 15/53). This means that, anti EBNA negative acute primary infection serologic profiles could be diagnosed as past infection, according to the anti EBNA IgG positivity. The IFA method is labor-intensive and requires experience. To prevent or minimalize the false positive or negative results, the slides were evaluated by two different researchers, and confirmed by a specialist in this topic. Besides, to make accurate comparison and to eliminate the differences between manufacturers, we used ELISA, IFA and immunoblotting kits from the same manufacturer. The antigens used by IFA for anti-VCA antibodies were antigen expressing P3HR1 cells and, native gp125 and recombinant p19 antigen. The antigens used by ELISA and immunoblotting were similar. For anti-EBNA IgG and anti-EA, ELISA and immunoblotting used recombinant antigens, but IFA used cells expressing EA antigen and Raji cells expressing EBNA.

In the active phase of the infection, EBV DNA is present in plasma or serum samples. In addition to active infection, EBV DNA viremia is present in reactivation, EBV associated malignancies, and posttransplant lymphoproliferative disease. Our study group consisted of pediatric patients; therefore the most common causes of the EBV serologic tests in routine practice were to diagnose the acute primary infection, and to exclude EBV infection in patients with lymphadenopathy. In our study, we detected EBV DNA in 9 of 16 acute primary infection patients. Therefore, we determined the sensitivity in active infection as 56.25%. EBV DNA was positive in 2 (viral loads were 6450 and 8730 copies/ml) of 9 late primary and 1 (viral load 92 copies/ml) of 127 past infection patients. The latter patient was a 3-year old child with classic triad of EBV infection as sore throat, cervical lymphadenopathy and fever. Interestingly, the stage determined by immunoblotting was as late primary infection with anti VCA IgM positivity. This finding made us think that whether ELISA and IFA methods could not detect the anti VCA IgM. But as IFA was considered as the gold standard test for the EBV infections, we suggested the anti-VCA IgM of immunoblotting as false positive. In routine practice, real time PCR would not be performed for this patient as it was considered as past infection. Detecting viremia by molecular methods is necessary for immunosuppressed individuals, as serological assays are insufficient due to their deficiencies in generating antibodies. Therefore, in immunosuppressed patients, detection of viral load by PCR is recommended (Hess, 2004). We suggest that the duration of viremia and the viral load may vary between patients, as we could not detect in some of acute primary patients but detected in late primary stage. Our positivity rate of EBV DNA in acute primary infection was lower than studies reported previously. Chan *et al.* (2001) and Gartzonika *et al.* (2012) reported the sensitivity of PCR in acute infections as 80% and 93.5%, respectively. The PCR system we used is a closed system that prevents the contamination, and the range of detection of the kit is $10–10^{10}$ copies/reaction. In the literature, it is reported that, in immunocompetent infectious mononucleosis patients, EBV DNA is detectable in serum for only approximately seven days after onset of symptoms (Fafi-Kremer *et al.,* 2005). In addition, Berth *et al.* (2011) reports that EBV DNA can be undetectable while the serological profile can be acute EBV infection. Therefore, we commented that the low sensitivity rate could be due to the short duration of viremia in our patients.

In our study, we aimed to evaluate the performances of different diagnostic methods on determining the infection stages and detecting the specific antibodies in serum samples of patients with suspicious of EBV infections. But as a limitation of our study, we did not evaluate the anti-CMV IgM and IgG antibodies to exclude cross reactivation on anti VCA IgM positive samples.

In conclusion, IFA is the gold standard test for the diagnosis of EBV infections, but the most important disadvantages are the high cost and the requirement of experienced staff. The compliance of ELISA with IFA is high, and this method provides advantages in terms of ease of use as it is practical and can be automated. Although immunoblotting is recommended as a confirmatory test in EBV diagnosis, high false positivity rates should be kept in mind. Especially in immunocompromised patients that serological methods are insufficient, real-time PCR is recommended to detect viremia. The most suitable diagnostic method should be decided according to the demographic characteristics of patient groups, experience of the staff, fin ancial facilities, working conditions of the laboratory, and when necessary the correct conclusion should be achieved using more than one diagnostic method as a test battery.

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