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Original Article

A hospital based pilot study on Epstein-Barr virus in suspected infectious mononucleosis pediatric patients in India

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Abstract

Introduction: Infectious mononucleosis (IM) caused by the Epstein-Barr virus (EBV) is commonly diagnosed by detection of antibodies in the patient's sera. Differentiation of acute from chronic and differential diagnosis of EBV-induced IM from IM-like syndrome caused by human cytomegalovirus (CMV) is important. The objective of this study was to standardize and use polymerase chain reaction (PCR) for diagnosis of EBV and evaluate it against enzyme-linked immunosorbent assay (ELISA).

Methodology: ELISA for detection of IgM and IgG antibodies to viral capsid antigen (VCA) and PCR targeting the VCA and EBNA1 gene of EBV and *mtrII* gene of CMV were performed on180 peripheral blood samples collected from 180 patients with suspected IM. The analytical sensitivity of PCR was evaluated against that of ELISA.

Results: Using the standard serological profile as the reference, the EBV-VCA gene was detected in 41 (95%) of 45 samples collected from patients with early primary infections, in 41 (54%) of 75 with recent primary infections, and in7 (17%) of 39 with past infections. The result of VCA PCR was statistically significant in virus detection during early or primary stage of infection. Nineteen (49%) EBV-seropositive samples were positive for CMV by PCR. All control samples tested negative for both VCA and EBNA1by PCR.

Conclusions: VCA PCR is sensitive for the detection of EBV DNA in the early or primary stage of infection and can be considered a reliable method to rule out the cross-reactivity and differential diagnosis of EBV-induced IM from IM-like syndrome.

Key words: infectious mononucleosis; Epstein-Barr virus; polymerase chain reaction.

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Introduction

Infectious mononucleosis caused by the Epstein-Barr virus is one of the most common infections reported among children and adults, followed by human cytomegalovirus. In India, very few publications are available on EBV-associated IM. The Epstein-Barr virus is contagious, as it can be contracted through direct contact with an infected person's saliva. About 95% of the population has been exposed to this virus by the age of 40, but only 15%–20% of teenagers and about 40% of those adults exposed to the virus become infected [1].

Antiviral agents, namely acyclovir or valacyclovir, are recommended to treat children with severe infection or those who develop complications [2].It has also been reported that patients with lymphoma and leukemia may present with clinical symptoms suggestive of infectious mononucleosis [3]. The most commonly used diagnostic criterion is demonstration of atypical lymphocytes in peripheral blood smear. However, presence of atypical lymphocytes alone is not sufficient for confirmation of mononucleosis. Further serological confirmation by demonstrating the presence of IgG and IgM antibodies produced against different EBV antigens/CMV is essential [4]. When IgM class of antibody to viral capsid antigen (VCA) alone are detected, an early primary infection is suspected; when IgG class of antibody to VCA alone are detected, a past infection is suspected; whereas, when both IgM and IgG positivity occurs, a recent infection or reactivation is suspected [5]. During early primary infection, IgM class of antibody to Epstein Barr viral capsid antigen is detected, whereas detection of IgG to VCA is seen in past infections and both IgM and IgG antibodies are detected during recent infection or reactivation.

Although serological investigations are preferred for diagnosis of CMV/EBV infection where the result of EBV serology presents a high degree of variability, serology provides rational criteria for interpretation of

the results [6]. The detection of antibodies is less useful in immunocompromised patients because of their immune system dysfunctions, and because the type of antibody and its maintenance may vary over time depending on the dynamics of the disease, thus leading to atypical profiles [7].

In the current study, polymerase chain reaction (PCR) targeting VCA designed by us and PCR targeting EBNA were evaluated against serological methods in the diagnosis of IM caused by EBV. The comparative applicability of both the methodologies to distinguish acute and latent EBV infection was determined. A correlative analysis of laboratory diagnosis and clinical presentations was done.

Methodology

The study was conducted at L& T Microbiology Research Centre, Vision Research Foundation, Chennai, after approval from the institutional research and ethical board (IRB) was obtained.

Patients and clinical specimens

Sixty peripheral blood samples were collected from immunocompetent healthy volunteer donors and served as controls. Donors were between 17 and 20 years of age. A total of 180 peripheral blood samples were collected between August 2010 and July 2013 from180 immunocompetent patients clinically suspected to have infectious mononucleosis at a tertiary child care center in Chennai. The ages of the patients ranged from 2.5 months to 14 years. All patients had EBV-related symptoms, namely fever, rash, lymphadenopathy, pharyngitis, hepatomegaly, or splenomegaly. Children with EBV-associated malignant diseases such as malignant lymphoma or chronic active infections were excluded.

Samples were collected after getting the informed consent from the patient or patient'sguardian. Clinical details were recorded by the clinician in the proforma made specifically for the study.

Two to three milliliters of the peripheral blood were collected in EDTA anticoagulated vacutainers and transported to a cold environment within four hours of collection. At the laboratory, the specimens were processed under sterile conditions for separation of plasma from the EDTA vacutainer, followed by separation of Buffy coat. The specimens were stored at -20°C. In addition, 2 mL of blood was collected in a vacutainer without any anticoagulant. Serum samples were separated and stored at 4°C until they were assayed. The serological and PCR tests were carried out in batches.

Enzyme immunoassay (EIA)

Human IgG and IgM antibodies against EBV-VCA were tested by EIA following the manufacturer's instructions (Demeditec Diagnostics, Kiel, Germany).All the samples were tested in duplicate. The test was also carried out in duplicate. The patients who had detectable IgM antibodies to VCA and an absence of VCA-IgG were considered to have early primary infection. Past infection was defined as a positive assay for IgG to VCA and negative for IgM to VCA. Recent infection or reactivation was defined as a positive assay for both IgM and IgG to VCA, and a negative assay for both IgM and IgG was defined as no EBV infection [5].

DNA extraction

Leucocytes of the Buffy coat suspended in 100µL of plasma were subjected to DNA extraction following the manufacturer's instructions (QIAGEN DNA extraction kit, Hilden, Germany). The extracted DNA was amplified for the detection of genes coding for VCA and EBNA1.

Semi-nested amplification of the EBV-VCA gene

The semi-nested PCR for detection of the EBV-VCA gene was standardized using the EBV standard strain culture infiltrate of marmoset cell line infected with EBV B958 (National Eye Institute, Bethesda, USA). Briefly, 10 µL of positive control DNA elute was subjected to amplification of the EBV-VCA gene. Primers targeting the VCA gene were designed using Primer Premier (Premier Biosoft International, Palo Alto, USA) based on consensus sequence obtained with VCA sequences of EBV submitted in GenBank. The nucleotide sequences of the primers and the expected respective product size are shown in Table 1. The nucleotide location of the first-round primers on the genome is 77002-77174, and that of second-round primers is 77058-77174.All primers and PCR reagents were procured from VBC Biotech Service (Vienna, Austria).

Optimization of VCA PCR

Both VCA and EBNA1 PCR were optimized to be carried out in the same thermal profile through gradient PCR temperature profile. The PCR mixture (50 µL) contained 100 mM of dNTP mixture, 10X PCR buffer with 15mM $MgCl_2$, 1 µM of each forward and reverse primer, and 3U/µL Taq DNA polymerase. Ten microliters of extracted positive control DNA was added to the first-round PCR reaction mixture. The reaction mix was incubated in the thermal cycler as

follows: denaturing the DNA at 94°C for 5 minutes followed by amplification for 30 cycles, secondary denaturation at 94°C for 1 minute, annealing at 59°C for 1 minute and extension at 72°C for 1 minute, with final extension for 7 minutes at72°C. For the second-round amplification, 5µL of the first round product was added to 50µL of the PCR mix containing 10 mM of each dNTP, 10X buffer, 1 µM of each forward and reverse primer, and Taq DNA polymerase. The PCR amplification was carried out for 20 cycles with the same thermal profile as mentioned above. PCR products were analyzed by2% agarose gel electrophoresis. Two controls (one reagent control and one reaction control) were included in each PCR run. The PCR results were considered valid only when the reagent controls were negative and the specific amplified product was obtained with the positive controls. To prevent contamination ofDNA extraction, PCR cocktail preparation, amplification, and analysis of results were carried out in physically separated rooms. Visualization of PCR product was done by subjecting 10 µL of amplified reaction mixture to electrophoresis on a 2% agarose gel incorporating 5 µg mL-1 of ethidium bromide in 1X Tris-Borate buffer (pH -8.2–8.6) and was documented on a gel documentation system (VilberLourmat, France).

Specificity of PCR for the detection of EBV-VCA gene

Specificity of the primers was determined against DNA extracted fromherpes simplex virus 1 (ATCC VR 733), herpes simplex virus 2(ATCC753167), Cytomegalovirus(ATCC 169),varicella-zoster virus(ATCC Oca strain), *Chlamydia trachomatis* (ATCC VR 341), *Toxoplasma gondii* (ATCC 50869), human DNA (extracted from whole blood), eubacteria (*Propionibacterium acne* lab isolate), and fungus (*Candida albicans*) (ATCC 90028). All the standard strains are maintained in the laboratory.

Sensitivity of PCR in detecting the EBV-VCA gene

DNA was extracted from 200 µL of EBV standard strain culture infiltrate of marmoset cell line infected with EBV B958 (National Eye Institute, Bethesda, USA); the concentration was primarily quantified using nanovue (GE Healthcare, Chennai, India). Serial tenfold dilutions of the DNA were made from 10^{-1} to 10^{-10}(*i.e.*, 5µL of DNA with 45µL of Milli-Q water). From each dilution, 10µL of DNA was taken for PCR reaction. The lowest dilution showing PCR positivity and its corresponding DNA concentration was calculated and considered as the sensitivity of the PCR.

Nested amplification of the EBNA1 gene [8]

Nested PCR was optimized and performed for all the samples collected targeting Epstein-Barr nuclear antigen 1(EBNA1). The primers used for the nested PCR amplification are listed in table 1.

Nested amplification of the mtrII gene of CMV [9]

Nested PCR for detection of CMV was performed on all samples collected targeting the *mtrII* gene of CMV. The primers used for nested PCR amplification are listed in Table 1.

Statistical methods

All the statistical analyses were carried out using Statistical Package for Social Sciences (SPSS) software version 14.0.Clinical sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and P value of VCA PCR were determined. A p value < 0.05 was accepted as statistically significant.

Table 1. List of primers used for amplification of genes that code for VCA, EBNA1 of EBV and mtrII gene of CMV

S. No.	Primer	Primer Sequence (5'-3')	Expected base pair
VCA	EBV F I	TTTGGCGTCTCAGGCTAT	Round 1 : 172
	EBV PP R	CGTGGTCGTGTTCCCTCA	
	EBV PP F	CGGTGTAACTACCCGCAATG	Round 2: 116
	EBV PP R	CGTGGTCGTGTTCCCTCA	
EBNA1	EBV up	GCAGTAACAGGTAATCTCTGG	Round 1 : 490
	EBV low	ACCAGAAATAGCTGCAGGACC	
	EBV up (R)	GATTTGGACCCGAAATCTGA	Round 2: 336
	EBV low (R)	CCTCCCTAGAACTGACAATTGG	
mtrII	MTR 1	CTG TCG GTG ATG GTC TCT TC	Round 1 :234
	MTR 2	CCC GAC ACG CGG AAA AGA AA	
	MTR 3	TCT CTG GTC CTG ATC GTC TT	Round 2: 168
	MTR 4	GTG ACC TAC CAA CGT AGG TT	

VCA: viral capsid antigen; EBV: Epstein-Barr virus

Results

Serology

A total of 180 specimens from patients suspected to have infectious mononucleosis were tested for the detection of IgG and IgM antibodies against VCA antigen of EBV by ELISA. Of 180 samples, 45 (25%) tested positive only for IgM antibody, 75 (41.6%)tested positive for both IgG and IgM, 39 (21.6%) tested positive only for IgG, and 20tested negative for both antibodies. Four samples (2.2%) tested positive for CMV IgG and fifteen (8.3%) tested positive for both CMV- IgG and IgM. Among 60 control sera from the healthy population tested, IgM antibody to VCA was detected in 9 samples (15%), and IgG antibody to VCA in was detected in 35 samples (58%).

Polymerase chain reaction

The primers used for VCA and EBNA1 PCRs were sensitive to detect 0.001ng DNA and were specific for EBV alone. Among the 180 samples that were processed, 89 (50%) tested positive for the EBV-VCA gene, and 84 (47%) were positive for theEBNA1 gene. Eighteen (30%) control samples collected from healthy donors were positive for EBNA1 PCR. None of the 44 healthy EBV-seropositive blood donors (controls) had detectable genes encoding EBV VCA. Nineteen samples (10.5%) tested positive for the CMV-*mtrII* gene.

Comparative study of EBV ELISA and PCR results

Among the 180 samples processed, 75 samples in which both VCA IgG and IgM antibodies were detected 50 (66%) tested positive for EBNA1, and 41 (54%) tested positive for EBV-VCA PCR. Of the 45 other samples in which only IgM antibodies were detected, 10 (22%) were positive for EBNA1 PCR, and 41 (95%) were positive for EBV-VCA PCR. Of 39 other samples in which only IgG antibodies were detected, 24 (61%) were positive for EBNA1 PCR, and 7 (17%) were positive for EBV-VCA PCR. IgG- and IgM-negative samples remained negative for both PCRs (Table 2).

The VCA PCR had clinical sensitivity of 69%, specificity of 67%, a positive predictive value (PPV) of 50%, and a negative predictive value (NPV) of 50% for diagnosis of early primary EBV infections. The p value for VCA PCR was calculated using SPSS and was found to be <0.001, suggesting high significance.

Correlation of EBV and CMV ELISA and PCR results

The EBV/CMV cross-reaction as the reason for false-positive EBV IgM reactions was confirmed by performing CMV PCR and ELISA.For better understanding and analysis of serological and PCR results, the patients were categorized into four groups (Table 3).Samples that were seropositive for only IgM antibodies to VCA and tested negative by both EBV PCRs belonged to group A (n=4). Samples that were seropositive for IgM and IgG antibodies to VCA by ELISA and also positive for VCA and EBNA1 PCR

Table 2. Correlation of EBV serological profiles with viral DNA detection in sample by PCR

S. No	Serology (N = 180)	EBNA 1 PCR [no. of patients positive / no. tested] (% positive)]	EBV VCA PCR [no. of patients positive / no. tested] (% positive)]
1.	Early primary infection IgG-IgM+ (n = 45)	10/45 (22)	41/45 (95)
2.	Recent infection/reactivation IgG+IgM+ (n = 75)	50/75 (66)	41/71 (54)
3.	Past infection IgG+IgM- (n = 39)	24/39 (61)	7/39 (17)
4.	No infection IgG-IgM- (n = 21)	0/21 (0)	0/21 (0)

EBV: Epstein-Barr virus; PCR: polymerase chain reaction; VCA: viral capsid antigen

Table 3. Correlation of EBV, CMV, ELISA, and PCR results on clinical samples

No. of samples	ELISA for EBV-VCA		ELISA for CMV		PCR		
	IgM	IgG	IgM	IgG	VCA	EBNA1	CMV
Group A (n = 4)	Positive	Negative	Negative	Positive	Negative	Negative	Positive
Group B (n = 20)	Positive	Positive	Negative	Negative	Positive	Positive	Negative
Group C (n = 15)	Negative	Positive	Positive	Positive	Negative	Negative	Positive
Group D (n = 21)	Negative	Negative	Negative	Negative	Negative	Negative	Negative

EBV: Epstein-Barr virus; CMV: cytomegalovirus; ELISA: enzyme-linked immunosorbent assay; PCR: polymerase chain reaction; VCA: viral capsid antigen

were categorized into group B (n=20). Group C (n=15) consisted of samples that tested positive for the IgG class of antibodies to VCA and negative for both PCRs. Group D (n=21) included samples that were both seronegative and PCR negative for detection of EBV.

Four samples belonging to group A tested IgG positive for CMV ELISA and were also found to have CMV DNA by PCR. Both CMV ELISA and PCR were negative in20other clinical samples belonging to group B. CMV PCR and both IgM and IgG antibodies against CMV were positive in samples belonging to group C. Twenty-one other samples categorized under group D tested negative for CMV by both ELISA and PCR (table 3). The CMV PCR results were reproducible in all samples tested positive and negative.

Clinical signs and symptoms

All the 180 patients included in the study were suspected to have infectious mononucleosis with presentation of high-grade fever and cough, and all the patients included had shown one of the criteria of infectious mononucleosis, namely lymphocytosis or the presence of atypical lymphocyte in the blood smear study. Among the 180 patients, 45 seropositive (VCA IgM positive) patients presented with decreased oral intake, pharyngitis lymph adenopathy, hepatosplenomegaly, abnormal liver function test, and/or rhinorrhea, in addition to fever and cough. The most common clinical symptoms observed among patients with early primary infection were high-grade fever and decreased oral intake.

Discussion

Though serological tests are considered a reliable tool by clinicians in the diagnosis of infectious mononucleosis caused by EBV, these tests do have demerits. IgM titer declines or disappears within four weeks of infection. EBV infection can be mistaken for CMV, streptococcal, or throat infection or an ordinary fever, strictly because of the symptom overlap.

Chan *et al*. concluded that neither a test of EBV VCA IgM nor a test of the presence of VCA IgG in the absence of EBNA1 antibody is reliable for diagnosing primary EBV infection. PCR for EBV DNA in plasma or serum is a useful addition to the panel of tests available for this purpose, particularly if used as a confirmatory test in conjunction with serological tests [10].

Using the standard serological profile as the referenced gold standard, the EBV-VCA gene was detectable in 41 (95%) of 45 patients diagnosed with clinical symptoms suggestive of early primary infections, 41 (54%) of 75 with recent primary infections, 7 (17%) of 39 with past infections, and none (0%) of 21 with no infection. Based on our study, it can be concluded that the PCR remains negative in seropositive patients and seropositive healthy donors in the absence of active EBV infection. The EBV/CMV cross-reaction as the reason for false-positive EBV-VCA IgM reactions was confirmed by performing PCR targeting the *mtrII* gene of CMV. CMV was detected by PCR in four samples found positive for EBV IgM antibodies and negative for EBV VCA PCR .Four EBV seropositive samples in which IgM antibodies to VCA were detected and EBV-VCA PCR was negative, were tested positive for CMV PCR. Therefore, in the current study, the standardized VCA PCR helped to detect EBV false IgM seropositive results.

The comparison of clinical data of patients diagnosed with early primary EBV infection based on PCR assay and serology revealed that high-grade fever and cough were observed in all groups. The study patients' symptoms included decreased oral intake, pharyngitis, lymph adenopathy, hepatosplenomegaly, abnormal or altered liver function, and rhinorrhea; however, these symptoms were observed only among the 45 seropositive (VCA IgM positive) patients(with early/primary infection)in addition to fever and cough observed in other patients. Forty-one among these 45 patients were positive for VCA PCR. No significant correlation of clinical symptoms was observed in the rest of the population, signifying that VCA PCR is highly sensitive and specific for the differential detection of EBV DNA in the early or primary stage of infection and can be considered a reliable method to rule out the cross-reactivity and differential diagnosis of EBV-induced infectious mononucleosis from infectious mononucleosis-like syndrome.

The limitations of the present study are mainly related to the study group. If the samples collected from both controls and patients belonged to same age group, the results obtained would better help to explain the seroprevalence among a healthy population when compared with an infected population. The tests should be done on follow-up samples to further understand the mechanism of infection and disease.

The merit of this study is that the standardized PCR targeting EBV helped in the early diagnosis of infection caused by EBV and this study proved that PCR could differentially diagnose the infectious

agents in patients having antibodies to both EBV and CMV.

Conclusions

VCA PCR is sensitive for the detection of EBV DNA in the early or primary stage of infection and can be considered a reliable method to rule out the cross-reactivity and differential diagnosis of EBV-induced IM from IM-like syndrome.

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