

Original Article

Investigation of ELISA and PCR for Diagnosis of Infectious Mononucleosis

Shirin Taleifard^{1*} M.Sc., Rasoul Roghanian¹ Ph.D., Majid Buzari¹ Ph.D.,
Hasan Salehi² Ph.D.

¹ Biology Department, Faculty of Sciences, University of Isfahan, Isfahan, Iran.

² Infectious Diseases Department, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran.

ABSTRACT

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Key words

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Background and Aims: Infectious mononucleosis (IM) is the clinical manifestation of primary infection with Epstein-Barr virus (EBV). Humans are the only known reservoir of EBV. Regarding the problems in diagnosis of the disease, the purpose of this study was to assess Enzyme-linked immunosorbent assay (ELISA) and Nested polymerase chain reaction (PCR) as a diagnostic tool for this disease.

Materials and Methods: 50 samples were collected from the suspicious patients with EBV and 50 samples from the healthy individuals as the control and both techniques were applied for them.

Results: The results showed that 76% of the patients and 14% of the control samples had EBV DNA in serum with PCR. Statistical analysis showed significant difference between the patient and the control samples for infection with EBV ($P < 0.0001$). Samples were classified into three groups according to the ELISA that were acute phase (20%), recent infection or convalescence phase (14%) and past infection (66%), respectively.

Conclusions: Comparing the two methods, the results of the ELISA test indicated that ELISA would be the best method to be used for the diagnosis of IM. Our results suggest that serology may be more sensitive and could be performed as the initial screening test for acute EBV infection. Although, the PCR test is routinely used as an accurate method for detection of the pathogens with a higher specificity and sensitivity comparing the immunoassay, in IM, ELISA seems to be the best method for detecting antibodies against EBV.

Introduction

Infectious Mononucleosis (IM) is an acute, self-limited, lymphoproliferative disease caused by the Epstein-Barr virus (EBV) [1]. EBV has a worldwide distribution being able to establish a lifelong infection in more than 90% of individuals [2]. Common symptoms of IM include sore throat, fever, pharyngitis, lymphadenopathy and splenomegaly [3]. Diagnosis of IM is based on clinical symptoms, haematological tests and serological testing [4]. The humoral response includes antibodies against antigens of both the lytic and latent phase [5]. After infection the virus persists latently in the host for life. Like other herpesviruses, EBV reactivates periodically in its host as a means of infecting new B lymphocytes as well as new individuals. The site of long-term persistence is the resting memory B cell. In the latently infected host a roughly constant number of infected B cells circulates in the peripheral blood; however, this number varies considerably between individuals [6].

The serologic responses in primary infection, such as IM, are characterized by the sequential appearance of immunoglobulin (Ig) M antibodies to viral capsid antigen (VCA), followed by the appearance of IgG antibodies to VCA and early antigen (EA). Antibodies to EBV nuclear antigen (EBNA) appear late sometime after several months. They are conventionally detected by Enzyme-linked immunosorbent assay (ELISA) [7]. Detection of IgM antibody to VCA in the absence of antibody to EBNA is regarded as suggestive of

acute primary EBV infection because EBNA antibodies develop only in late convalescence [8, 9]. In previously infected (seropositive) individuals, VCA and anti-EBNA IgG antibodies are always present. Immunologic deficiencies permit productive cycles of EBV replication, which leads to increased production of VCA IgG antibodies. EBNA, EA, VCA and latent membrane protein are the major antigens of the virus. IgM antibodies produced against VCA indicate acute infection and are detectable for approximately 3 months. IgG antibodies could be identified 4-7 days after the start of symptoms and persist lifelong. EBNA IgG antibodies indicate the convalescent period and may persist throughout a person's lifetime [10]. Moreover, a variety of molecular diagnostic methods, primarily based polymerase chain reaction (PCR), have been developed to detect and quantify circulating EBV in an effort to predict or detect the onset of EBV associated disorders and to assess the efficacy of therapeutic intervention [11]. PCR has been used to detect EBV-DNA in cell free serum or plasma samples of patients with primary and persistent EBV infection [4]. Regarding the point that immediate diagnosis of EBV infection is important to prevent severe complications in patients, we wanted to investigate which method can be used to immediately and reliably diagnose EBV infection. In the present study, a comparison between ELISA and molecular technique (PCR) was performed to provide better

understanding of more accurate and suitable diagnostics tool for patient with IM.

Materials and Methods

Patients and samples

EDTA-anticoagulated whole blood of 50 patients who had symptomatic EBV infection (35 women and 15 men) was collected from medical laboratories (Bradaran and Ferdowsi laboratories, Isfahan, Iran). These specimens were received for clinical testing. Positive diagnosis was based on an ELISA assay for EBV antibodies and mono test for heterophile antibodies. Symptoms at presentation to the clinic included fever, sore throat, lymphadenopathy and fatigue. The age of patients ranged from 6 to 60 years. In addition, 50 serum samples collected from healthy blood donors as negative control. Ethical clearance was taken from institutional ethical committee.

ELISA assay

Patients' sera were assayed to determine VCA IgG, VCA IgM, and EBNA IgG using commercial ELISA kits (EUROIMMUN, Medizinische, Labordiagnostika, Germany) according to the manufacturer's instructions. Results of the VCA IgM, VCA IgG, and EBNA-1 IgG antibody assays were classified according to their index value as negative (<0.80), equivocal (0.80-1.1), or positive (0>1.10). IM was diagnosed by clinical findings and serological examinations as follows: positive for anti-VCA IgG and/or IgM and negative for anti-EBNA antibody.

DNA extraction

For the PCR assay, DNA was extracted from the clinical sample materials using an alkaline phenol-chloroform isoamyl alcohol procedure. Briefly, 200 µl of specimen was placed in 12.5 µl of proteinase K solution (65 mg/ml) (Sigma-Aldrich Corp, St.Louis, MO, USA) for 2 hour at 65°C., DNA was extracted from the supernatant using a mixture of 125 µl alkaline phenol and 125 µl chloroform-isoamyl alcohol (24:1). DNA was washed in 75% ethyl alcohol at 13,000 g for 2 min at 4°C, air-dried at 37°C and dissolved in 100 µl distilled water. The serum DNA samples were stored at -20°C until needed.

PCR assay

Four micro liter of DNA elute was used for a first PCR amplification with a 10 pmol concentration of each sense and antisense primer (5'- AAG GAG GGT GGT TTG GAA AG-3'and 5'- AAC AGA CAA TGG ACT CCC TTA G-3'), respectively, corresponding to the EBNA-1 gene of EBV to detect EBV DNA. The PCR mixture (25 µl) contained PCR buffer (10 mM Tris-HCl, 2.5 mM MgCl₂, 50 mM KCl, 0.1% gelatin [pH= 8.3], 10 mM deoxynucleoside triphosphate and 1 U of Taq polymerase (Cinagen, Iran). Samples were then subjected to cycles of amplification (5 min. at 94°C, 30 s at 94°C, 30 s at 56°C and 45 s at 72°C) in thermal cycler (Eppendorf, Germany) followed by extension at 72°C for 5 min. Subsequently, a 2 µl aliquot of the first PCR product was transferred to a second PCR tube for nested PCR using two inner primers (5'-ATC GTG GTC AAG GAG GTT CC-3'

and 5'-ACT CAA TGG TGT AAG ACG AC-3') [12], each at a 10 pmol concentration. The PCR mixture was used similar to that of the first PCR. The reaction mixture was subjected to a further 35 cycles of amplification using the thermal cycling profile described above. PCR products were detected in 2% ethidium bromide-stained agarose gel electrophoresis. The size of the nested PCR product was 208 bp (Fig.1). In each experiment, a negative control being composed of either sterile water instead of genomic or serum human DNA as well as a positive control EBV DNA was tested. In order to demonstrate the presence of DNA in negative samples, specific primers were used for β globin gene which is detectable in all eukaryotic cells. The sequence of β globin primers were (5'- GAA GAG CCA AGG ACA GGTAC- 3' and 5'- CAA CTT CAT CCA CGT TAC ACC- 3'), respectively [13]. The positivity of the samples was assessed by the standard agarose analysis.

Statistical analyses

Statistical analysis enabled us to measure the agreement of the two parameters, i.e., positive EBV-DNA PCR in serum samples and

positive EBV antibodies in acute phase of EBV infection. For this reason, Fisher's exact was applied (SPSS 18.0). P value less than 0.05 were considered statistically significant.

Results

In this study fifty subjects (35 women and 15 men) participated. The median age of the patient's samples was 33 years (range 6-60 years). Stages of EBV infection were confirmed by detecting EBV antibody in all of the samples. Ten (20%) subjects were categorized as having acute EBV infection of positive for VCA IgM antibody and negative for EBNA IgG antibody. Seven (14%) subjects had evidence of recent infection because they were positive for VCA IgG antibody and negative for VCA IgM and EBNA IgG antibodies. Thirty three (66%) subjects had evidence of previous EBV infection because they were negative for VCA IgM antibody but positive for both VCA IgG and EBNA IgG antibodies.

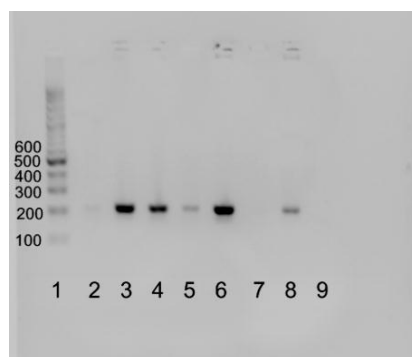


Fig.1. Analysis by agarose gel (2%) electrophoresis of the PCR products. The 208bp DNA corresponds to Specific Epstein - Barr virus DNA sequence. Lane 1: 100bp ladder. Lane 2 and 7: negative sample. Lane 3,4,5,6 and 8: products of positive samples. Lane 9: negative control.

Table 1. Correlation of EBV serological profiles with viral DNA detection in serum by PCR

Diagnosis based on standard EBV seroprofile	No. of patient s	EBV DNA in serum (No. of patients) (% positive)
Acute infection (VCA IgM ⁺ , EBNA IgG ⁻)	10	8(80)
Recent infection (VCA IgG ⁺ , VCA IgM ⁻ , EBNA IgG ⁻)	7	4(57)
Past infection (VCA IgG ⁺ , VCA IgM ⁻ , EBNA IgG ⁺)	33	17(51)

Thirty eight of fifty DNA samples extracted from serum were EBV-DNA positive. EBV DNA was detected in the serum in 8(80%) out of 10 samples diagnosed with acute infections, 4(57%) out of 7 samples with recent infection and 17(51%) out of 33 samples with past infection. EBV was detected in 7(14%) samples from the serum specimens of 50 healthy adults and 12 samples were seronegative for EBV infection. The results are shown in table 1. Amplification of human β -globin sequences rendered visible bands upon gel electrophoresis of all serum samples from patients with infectious mononucleosis and all serum samples from healthy individuals. Sequence analysis was performed on a subset of samples to confirm the results of PCR assay. The results showed that DNA extracted from patient samples has 98% similarity with human herpes virus 4 isolates.

Discussion

Primary EBV infection is usually asymptomatic in childhood whilst at times induces acute infectious mononucleosis in susceptible adolescents or adults. EBV has evolved a successful strategy of immune evasion without disturbing the immune homeostasis of the host [4]. Infectious mononucleosis is mainly the result of a prominent T-lymphocyte proliferation

occurring in response to EBV-carrying B lymphocytes [14]. Increased availability and use of molecular tests for detection of microorganisms have improved our ability to diagnose of infections but they are often used as the gold standard without consideration of potential limitations. A number of different methods, techniques and protocols have been used to determine the presence of EBV DNA and measure viral load [5]. Few studies attempted to characterize the relationship between quantitative PCR for Herpesviridae and their serologic parameters [15, 5, 16, 17]. PCR was performed to detect EBV DNA in the sera of patients with serologically defined EBV infection. The results driven from PCR showed that 38 (76%) of patient subjects had EBV DNA in serum. The percentage of positive samples in our report confirms the results of previous studies that approached the same values [8, 15, 17-19]. In this study, DNA of EBV was detected solely in 7 out of 50 samples of the healthy individuals whereas in 38 patients, samples were known seropositive and 12 seronegative. The presence of EBV DNA in only 7 subjects out of 38 seropositive healthy controls indicates that although most such individuals would be expected to be carrying EBV DNA in their lymphocytes, EBV DNA does not appear in serum in the

absence of active EBV disease, confirming previous reports [5,8]. In this study, 3 antigens of virus were investigated. Among fifth samples, 15 samples showed IgM positive for VCA protein, 38 samples IgG positive for VCA and 28 samples IgG positive for EBNA antigen. Assay for IgM VCA has often been used as an indicative assay for the diagnosis of primary EBV infection. Because this antibody is detectable in primary infection, it could be used as a marker for acute infection with EBV virus. Moreover, because this antibody is seen in recurrent infection with this virus, it is recommended that detecting of EBNA antibodies along with assays for VCA IgG and/or IgM for the diagnosis of primary EBV infection be used. A reliable test for EBNA antibodies might be used as a screening test, since the presence of EBNA antibodies excludes primary EBV infection. Antibodies against other EBV antigens must then be analysed only if EBNA antibodies are absent [20].

Conclusion

The findings from this study indicates that the search for EBV DNA may be more sensitive than serology in the early stage of the IM, and some studies have shown that it correlates better with clinical acute infection than the avidity of VCA IgG. The results of the analysis showed that during the acute phase, there was no significant difference between the ELISA and PCR. Because the load of virus is high in acute phase of infection, the viral genome specifically can be detected by PCR in

serum and whole blood. During the recent infection, there was no significant difference between ELISA and PCR. At this stage of infection, PCR method does not help to identify the disease in patients because the load of virus is falling in blood. In the past infection, the ELISA test is more sensitive and specific for titers of antibodies that remain in patients for life time. By comparing the two methods in this study, the results showed that although the PCR test is a sensitive for detection of pathogens, in IM, because of the ability of EBV to evade from immune system after two weeks of infection, PCR method is not economically cost effective for patients and also compared with serologic methods more time is required. Meanwhile, the ELISA test can be performed in a short time and with low costs in comparison with molecular types.

In conclusion, the ELISA technique is the sensitive and specific diagnostic method for mononucleosis infection disease and PCR may serve as a useful additional diagnostic tool for clarifying serological dilemmas, reaching final diagnosis and defining status of the infection.

Conflict of Interest

The authors declare that they have no conflict of interest.

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