

Detection of Epstein - Barr virus Using Polymerase Chain Reaction with and without DNA Extraction from Native Sera

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Original Research Article

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Article History

Received: 02.10.2018

Accepted: 18.10.2018

Published: 30.10.2018

DOI:

10.21276/haya.2018.3.10.6



Abstract: PCR has been commonly used for genomic viral diagnosis for its sensitivity and accuracy. It showed a higher sensitivity when compared to virus isolation in tissue culture and also in antigenemia detection. Definitely, DNA samples are critical factor in PCR validity. Out of 167 serum samples subjected to this study and extracted by Wizard® DNA purification mini kit, 47 samples (28.1%) were positive PCR. While, 21 samples (12.6%) only out of the same population study (167) were positive PCR for EBV DNA in native serum samples (without DNA extraction). This result was confirmed the importance of DNA extraction from serum samples for detection of EBV which, subsequently lead to more sensitive diagnostic tool of an ongoing EBV infection.

Keywords: EBV DNA, nested PCR, DNA extraction, serum.

INTRODUCTION

Epstein Barr Virus (EBV) is known to cause mild or asymptomatic infection in most healthy individuals but it can also cause EBV-associated lymphomas, lymphoproliferative disorders, hemophagocytic lymph histiocytosis, solid tumors, and other diseases in some immunosuppressed hosts [1].

The most commonly used method to detect EBV infections was, until recently, conventional cell culture on human fetal lung fibroblasts. The problem with this procedure is that cytopathic effect (CPE) evolves, most of the time, very slowly: it can take up to 21 days to visualize the CPE of EBV infection in cultured cells [2, 3].

In healthy carriers, EBV is mostly present in a latent form as episomal DNA in resting memory B cells. Therefore, only very highly sensitive polymerase chain reaction (PCR) assays can detect EBV-DNA in peripheral blood cells [4]. The conventional technique, using dextran sedimentation, seems to be time consuming: 5 hrs is required to obtain a result. Today, methods based on the direct detection of either the viral genomic DNA or the viral mRNAs synthesized at different times of the viral replication cycle are more and more currently used.

Nucleic acid amplification by PCR methods has become the most widely used diagnostic tool for EBV infection [5].

The diagnostic value of EBV DNAemia detected by PCR of leukocytes of immunosuppressed patients has been restricted by its low correlation to EBV viremia [5] and EBV disease [7]. Recent, studies have accepted PCR for detection of EBV DNA in

plasma [8, 9] and serum of immunosuppressed patients [10, 11] as a good tool for this purpose.

During recent years PCR protocols were applied for detection of numerous human viral and non-viral pathogens [12]. The ability to demonstrate the presence of viruses in clinical samples with unprecedented sensitive assays has been the driving force for using PCR as a novel diagnostic procedure. Problems are encountered, however, if PCR is employed for the detection of viruses that establish lifelong latency in the host interrupted by episodes of recurrences [13]. Thus, during acute infection with Epstein Barr Virus (EBV), viral DNA is readily detected by PCR in peripheral blood mononuclear cells (PBMCs), biopsies, serum, urine and various other specimens [14, 15], but positive results may be obtained during asymptomatic reactivations and in latently infected healthy individuals as well [13]. Therefore, without further laboratory and clinical data, positive PCR results are difficult to interpret. So, this study was aimed to determine the best clinical samples which give

the maximum validity and sensitivity of applied technique (nested PCR).

MATERIALS AND METHODS

Study population

A total of 167 serum samples were used for monitoring EBV. The samples were kindly supplied from clinical units of El Demerdash Hospital, El Menya hospital, and El Moqawelon Hospital, Cairo, Egypt. These samples were screened, in this study, for EBV DNA by nested PCR.

DNA extraction

EBV DNA was extracted from 300 µl serum sample using Wizard® DNA purification mini kit, Promega (Madison, USA), 300 µl serum was added to 900 µl Cell Lysis Solution (included in Wizard® DNA purification mini kit, Promega) and mixed well. After incubation at 30 °C for 10 min, the tubes were centrifuged at 11500 xg for 20 Sec at room temperature; the supernatant was removed without distributing the visible white pellet.

300 µl Nucleic lyses solution (included in Wizard® DNA purification mini kit, Promega) was added to the tube containing the white pellet, mix well, then incubated at 37 °C. 1.5µl of RNase solution (included in the same kit) was added and the sample was mixed by inverting the tube 2-5 times. The mixture was incubated at 37 °C for 15 min, and then cooled at room temperature. 100 µl protein precipitation solution (included in the kit) was added and vortex for 10-20 min. After centrifugation at 11500 xg for 3 min at room temperature, the supernatant was transferred to a clean 1.5ml microcentrifuge tube containing 300µl isopropanol, the tube was mixed well, then centrifuged at 11500 xg for 1 min. The DNA was visualized as a small white pellet. The supernatant was removed and one sample volume of 70% ethanol was added to the pellet, then the mixture was centrifuged at 11500 xg, and then the ethanol supernatant was removed. 100 µl DNA dehydrating solution (included in kit components) was added and DNA was rehydrated by incubating at

65 °C for 1h, DNA was left over night at room temp, and then stored at -20 °C until use.

Nested PCR

Nested PCR of serum samples for detection of EBV-DNA was carried according to [16]. The reaction mixture of the qualitative PCR contained, in total volume of 25 µl, 5 µl 10x buffer (10mM Tris-HCl pH 8.0, 50mM KCl, 25mM MgCl₂), 0.5 µl 50mM dNTP mix, 0.25 µl of primers E2P1: 5[\] ATCCTTGCACTTAGCCAAGC 3[\] and E2P2: 5[\] TCCAGATGTGTCTCCCTTCT 3[\] (Bioneer, Atlantic Avenue, Alameda, USA) for amplification of 556 bp fragment in EBNA-2 gene, 5 µl DNA solution (DNA template), 14.15 µl distilled water, and 0.1 µl 2U of Taq DNA polymerase (Bioneer, USA) were added. Nested PCR was performed according to the following thermal cycling protocol: pre-denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 sec, annealing at 57 °C for 30 sec, extension at 72 °C for 60 sec and final extension at 72 °C for 4 min. The second round of the nested PCR was conducted by using 2 µl of the 1st PCR product with the same reaction mixture as mentioned above. Except for internal primers Ap1: 5[\] CCAGTAGCATCTCTGTCTGG 3[\] and AP2: 5[\] GAACCATCCTCGTCCTCATC 3[\] (Bioneer, Atlantic Avenue, Alameda, USA) or amplification of 190 bp fragment in EBNA-2 gene, under the same thermal cycling protocol.

PCR Amplification of serum samples without DNA extraction:

1ml of human serum sample was boiled at 95 °C for 2 min, centrifuged at 11500 xg for 5 min. 5 µl of the supernatant was used as DNA template in the same nested PCR protocol that mentioned above.

RESULTS

Detection of EBV DNA by nested PCR

In DNA extracted from serum sample

Results in Fig-1 showed that 47 out of 167 tested samples gave positive EBV-DNA using nested-PCR technique for DNA extracted from serum samples.

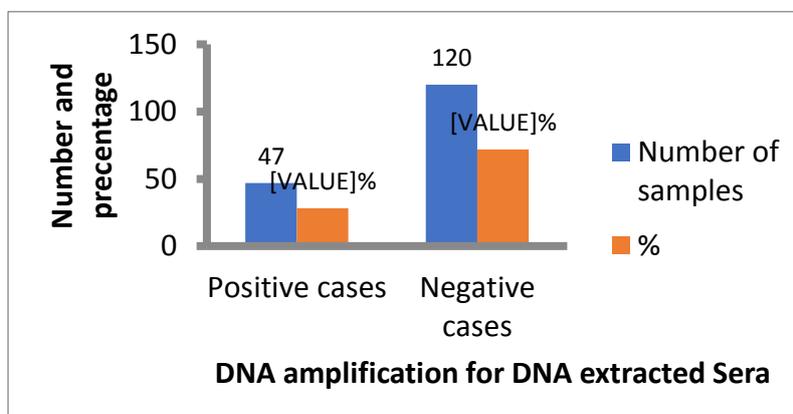


Fig-1: Detection of EBV-DNA from extracted DNA of serum samples using nested-PCR

In native serum samples

Results in Fig-2 showed that the native serum samples (without extraction of EBV-DNA) gave considerable reduction in the number of positive EBV

infection comparing with the previous cases (see Fig-1). Where 21 out of 84 tested samples (12.1%) were positive for EBV-DNA. The majority of cases were negative for EBV-DNA.

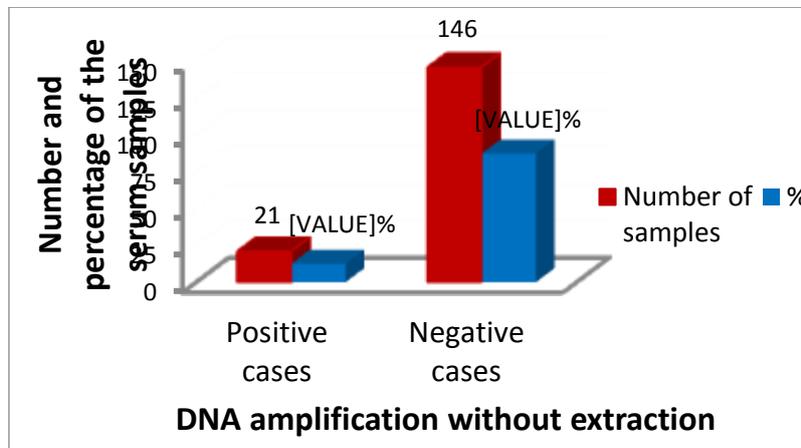


Fig-2: Detection of EBV-DNA in native serum samples using nested-PCR

Nested-PCR indicated that all tested positive EBV-DNA samples were also positive (100%) when DNA was extracted from serum samples. Whereas only 21/47 (45%) were positive when native serum samples were used without DNA extraction (Table-3). These

results confirmed the necessity of extraction of viral genome (DNA) before amplification by nested-PCR. Results in Fig-3 showed that the positive samples gave PCR-product at 100bp as an expected size of EBV-DNA fragment.

Table-3: Detection of EBV DNA in some selected samples showing DNA amplification using either DNA extraction kit or native unprocessed sera

Nested PCR	None (native Sera)	extraction (Wizard kit)
Positive	21 (45%)	47 (100%)
Negative	26 (55%)	0 (- %)

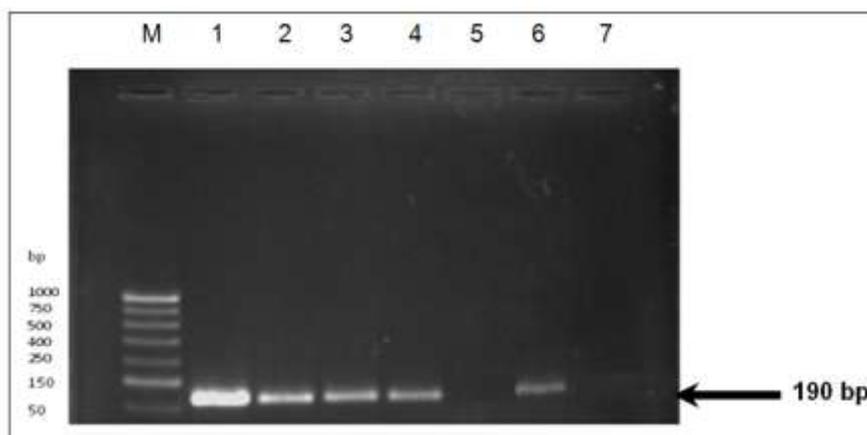


Fig-3: Nested PCR results of EBV DNA in serum samples Lanes 1, 2, 3, 4, 6 were positive for EBV DNA, Lanes 5, 7 were negative for EBV DNA. M= DNA marker

DISCUSSION

EBV is detected earlier by PCR than by other techniques [13]. The uniplex PCR for EBV DNA detection followed by confirmatory nested-PCR with AP primers has been used for processing urine, serum, plasma, saliva, milk and other secretion samples, showing high sensitivity and specificity. These

sensitivity and specificity rates are higher than those reported for EBV serological tests [17].

There is an urgent need for technical standardization of procedures used for isolation and amplification of DNA. It seemed that the influence of methodological aspects such as DNA extraction

procedures are important for the sensitivity and specificity of EBV DNA detection from cell free sera by PCR [7, 18].

Several techniques, using different clinical samples have been employed to detect EBV-DNA and CMV-DNA either by boiling serum samples without DNA extraction [19] or by using recently DNA commercial kits [2]. Two decade ago, Ishigaki *et al.*, [20] demonstrated that the detection of CMV-DNA in serum or plasma samples without any performance extraction of DNA has a disadvantage regarding the lower sensitivity of applied technique.

In this study, the percentage of positive nested-PCR of EBV DNA extracted from serum samples were higher than that of positive cases for EBV DNA without DNA extraction, indicating that the sensitivity of EBV DNA amplification from DNA extracted serum was higher than that of amplification from native sera samples. The false negative nested-PCR (44.4%) has been shown with the native serum sample. Whereas, no negative results was shown with the extracted EBV-DNA with Wizard® DNA purification mini kit. It is possible that serum components acted as non-specific, non-enzymatic PCR inhibitors which were not eliminated by the thermal inactivation, but were eliminated after Wizard extraction [11].

Since the sensitivity of PCR for the amplification of EBV DNA sequences in serum may often be reduced due to the presence of inhibitory factors. Still the method of DNA extraction has perfect effect to detect the target nucleic acid in clinical diagnostic samples [21].

Interestingly, the number of samples with positive EBV-DNA amplification in cases of DNA extracted sera was higher than that from native sera of the same cases. This may be attributed to the effect of Wizard® DNA purification mini kit in decreasing the effect of inhibitors on detection of EBV DNA in sera of these samples. Similar results were reported by Bevan *et al.*, [22], Brytting *et al.*, [23], Klaus *et al.*, [24] and Klein *et al.*, [25].

CONCLUSION

This study concluded that, detection of EBV DNA by nested PCR from native sera without DNA extraction was less sensitive than that by using DNA extraction kit. DNA extraction and purification methods had significant influence on the quality of DNA. DNA purification kits improved the accuracy of nested PCR results in clinical samples.

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