

PCR Based DNA Sequencing Targeting LMP, EBNA2 and EBNA3C Genes of Epstein Barr Virus for Genotyping

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Abstract:-Epstein Barr Virus is the causative agent of infectious mononucleosis which is commonly diagnosed by detection of antibodies in the patient's sera. The virus highly prevalent since it affects more than 90% of individuals Worldwide and has been linked to several malignancies which are common after transplantation. The objective of the research paper was to standardize PCR targeting genes that codes for EBNA3C, LMP and EBNA2 of EBV. Twenty Peripheral blood specimens were collected from 20 patients diagnosed to have infectious mononucleosis and proven already positive for EBV by PCR targeting VCA gene. All the PCR positive products were subjected to DNA sequencing with forward and reverse primers. Nucleotide sequence results were analyzed by BIOEDIT software and found to have 96-100% homology with Human herpes virus 4 strain B95.8. The genotyping and identification of molecular aspects of the virus leads to better understanding of the mechanism of action of proteins and their role in the development of different diseases. Using the standard serological profile as the reference, the standardized PCR targeting EBNA2, EBNA3C and LMP genes was carried out for 20 clinical specimens. Out of 20 clinical specimens 18 (80%) (Table 5) clinical specimens were positive for the EBV type1 with EBNA2, EBNA3C and LMP genes indicating a band at 378bp, 153bp and 160bp respectively.. All clinical specimens showed negative for the EBV type2 gene. Thus indicating that EBV type1 gene is most prevalent in the Chennai population. From my research I do summarize that EBV type 1 was found to be predominant in Chennai from the given blood sample which were positive for the infectious mononucleosis.

IndexTerms; Epstein-Barr virus, LMP-1 oncogene, EBNA2, EBNA3C.

I. INTRODUCTION

Epstein Barr Virus is a lymphotropic human gamma-1-herpes virus which is transmitted through saliva and infects 95% of the world's population, in the developing countries, Primary EBV infection which typically occurs in early childhood is asymptomatic, infants become susceptible to EBV as soon as maternal antibody protection presenting at birth disappears while in the developed countries infections occur in the later childhood or young adulthood and manifest as infectious mononucleosis⁽²⁾. The virus is ubiquitous in nature and infects B-lymphocytes and the epithelial cells, the primary site of Epstein-Barr virus (EBV) infection is the oropharyngeal cavity. Children and teenagers are commonly infected usually after oral contact, hence the name "kissing

disease". Based on serology, EBV remains lifelong carriers of the virus. In developed countries, exposure to EBV occurs relatively late: only 50–70% of adolescents and young adults are EBV seropositive^(3, 4).

The disease is characterized by fever, sore throat, generalized lymphadenopathy, splenomegaly, intense asthenia, hyper-lymphocytosis (>50%) with atypical lymphocytes and elevated transaminase levels^(5, 6). The EBV can be identified by both serological and molecular techniques; the most commonly used serological test is heterophile antibody test also known as the monospot test, western blot, flow cytometry and enzyme-linked immunosorbent assay can potentially detect the selected viral proteins in which antibodies are available while the molecular techniques involve PCR, insitu hybridization and microarray.

The knowledge of better understanding of LMP, EBNA2 and EBNA3 needed to evaluate prevalence of the virus in the population and how the oncogenic properties can be controlled. It also helps in the identification of new therapeutic targets that can be of great benefit in the developments of new antibiotics and antiretroviral. Therefore the current work was undertaken to study the genotypic prevalence of EBV among patients with infectious mononucleosis.

II. LITERATURE REVIEW

The Epstein Barr virus also known as the human herpes virus 4 (HHV-4), was discovered by Epstein and Barr in 1964.^(8, 9) It was later found to be the causative agent of infectious mononucleosis (IM) in 1968 by Henle since then many reports followed linking EBV to several other clinical complications⁽¹⁰⁾.

III. EBV LATENT GENE

Epstein-Barr nuclear antigen 1 (EBNA1) is a multifunctional, dimeric viral protein and was the first Epstein-Barr virus (EBV) protein detected. It plays a key role in establishing and maintaining the altered transformed cells by EBV, thus EBNA1 is required for gene regulation, replication and maintenance of viral genome in latency⁽¹⁵⁾.

EBNA1 is expressed in both latent and lytic modes of EBV infection, although it has mainly been studied in latency, where it plays multiple important roles. The importance of EBNA1 in

EBV latency is reflected in the fact that EBNA1 is the only viral protein expressed in all forms of latency in proliferating cells and in all EBV-associated tumors. EBNA1 is required for the persistence of EBV genomes due to its contributions to both the replication and mitotic segregation of EBV episomes⁽¹⁶⁾.

LMP1 is an integral membrane protein of 386 amino acids, is the only known gene with the tumorigenic potential for rodent fibroblast (wang et al.1985),it is located close to the right hand terminus of the linear viral genome , is transcribed leftwards and encodes an integral membrane protein of 386 amino acids , it is expressed in many associated tumors , it mimic the function of a key cellular signaling proteins CD40, whose role in the immune system is to induce survival and differentiation of antigen activated B cells to long lived memory cells^(22,23).

EBNA-3A and EBNA-3C are necessary for immortalization of the B lymphocytes; EBNA-3B is one of the primary targets for the recognition of immortalized cells by cytotoxic T cells. EBNA-3A,-3B, and -3C are members of a family of nuclear proteins composed of 944,937, and 992 amino acids residues respectively, they are encoded by tandem arranged genes located in the middle of the linear viral genome⁽²²⁾.

IV. METHODOLOGY

Clinical Specimens: Twenty Peripheral blood specimens was collected in EDTA vacutainer from 20 patients diagnosed to have infectious mononucleosis and proven already positive for EBV by PCR targeting VCA gene.

The PCR was carried out with the extracted DNA using the following PCR protocol. Primers targeting genes that codes for EBNA3C, LMP1 & LMP2 genes was designed using Primer premier biosoft international, USA based on consensus sequence was obtained with specific sequences of EBV specific genes submitted in GenBank. The nucleotide sequences of the primers and the expected respective product size are given in table 1. All primers and PCR reagents were procured from VBC – Biotech service, Vienna.

List of primers used for amplification of genes those codes for, EBNA1, EBNA2 and EBNA3C of EBV:

S. No.	Primer	PRIMER SEQUENCE (5'-3')
EBNA3C	NA3C-F	AGAAGGGGAGCGTGTGTTGT
	NA3C-R	GGCTCGTTTTTGACGTCGGC
LMP	LMP 1 F	TGAACACCACCACGATGACT
	LMP 1 R	ATTGTCAGGACCACCTCCAG
EBNA2	EBNA 2 F	5'TTTCACCAATACATGAACC3
	EBNA2 R	5'TGGCAAAGTGCTGAGAGCA3

The PCR positive -amplified products were further subjected to DNA sequencing to compare with the standard strain and to determine the homology percentage. Cycle

sequencing of the amplified products was performed in a 10-µL reaction volume, containing 0.5 µL of RR mix, 3.5 µL of sequencing buffer, 2 µL of forward primer (1:100 diluted), 2 µL MilliQ water, and 2 µL of amplified . Amplification was carried out in the Perkin- Elmer thermocycler using 25 cycles at 96°C for 10 s, at 50°C for 5 s, and at 60°C for 4 min, with initial denaturation at 96°C for 1 min. The cycle-sequenced products were then purified and sequenced using ABI Prism 3130 AVANT (Applied Biosystems, USA) genetic analyzer, which works based on the principle of Sanger’s dideoxy termination method. The sequences were then analyzed by Bio Edit sequence alignment software, (www.softpedia.com/progDownload/BioEdit-Download-174716) and BLAST analysis (www.ncbi.nlm.nih.gov/BLAST) was done.

V. RESULTS

The standardized PCR targeting EBNA2, EBNA3C and LMP genes was carried out for 20 clinical specimens. Out of 20 clinical specimens 18 (80%) (Table 5) clinical specimens were positive for the EBV type1 with EBNA2, EBNA3C and LMP genes indicating a band at 378bp, 153bp and 160bp respectively.. All clinical specimens showed negative for the EBV type2 gene. Thus indicating that EBV type1 gene is most prevalent in the Chennai population.

Table 1: showing the results for clinical samples.

PCR	EBV Type -1	EBV Type-2
EBNA2 PCR	18	0
EBNA 3C PCR	18	0
LMP PCR	18	0

Figure 2: agarose gel electrophoresis showing amplification of EBNA2

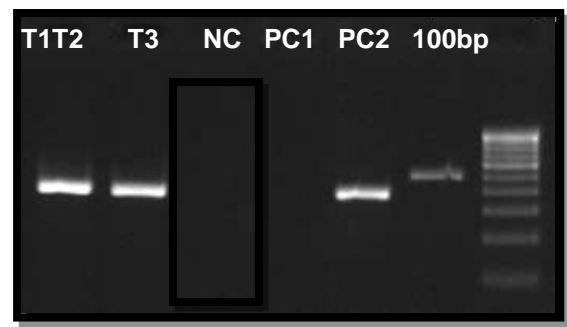


Figure 3: agarose gel electrophoresis showing amplification of EBNA3C

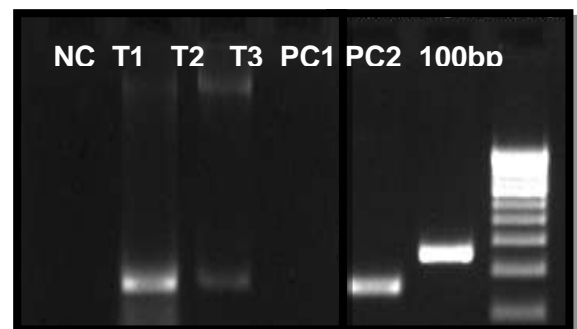
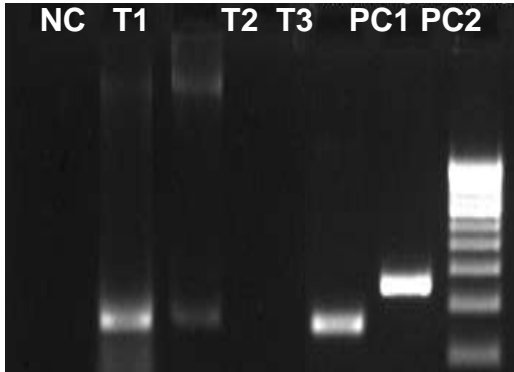


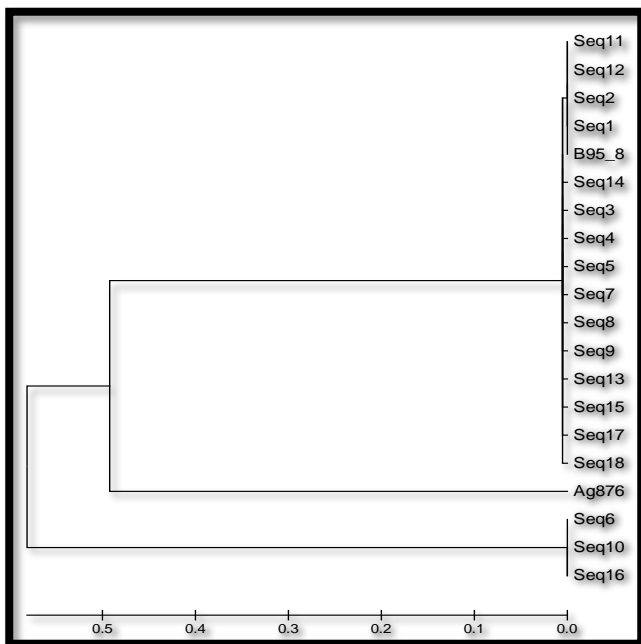
Figure 3: agarose gel electrophoresis showing amplification of LMP



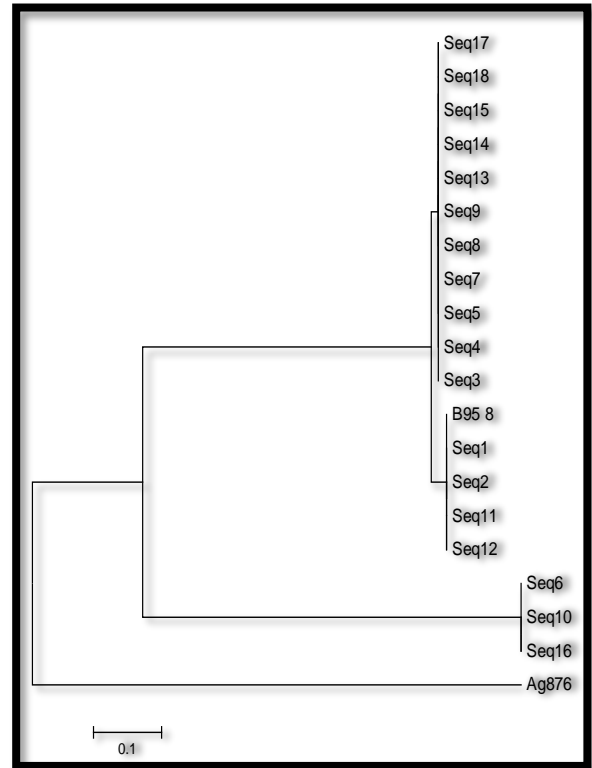
NC-Negative control, Lane 1-3(T1 – T3): DNA extracts from samples
 T1 : EBV 1 Positive T2: EBV 1 Positive, T3 : Both types negative
 PC1: positive control, DNA extracted from type 1 standard strain - B95-8
 PC2: positive control, DNA extracted from Type2 standard strain - Ag876
 100Bp: Molecular weight marker (100-1000bp ladder)

The nucleotide sequences of the EBNA2 and EBNA3C PCR positive amplified products were analyzed by comparison with EBV standard strains nucleotide sequences. The sequences were aligned using BIOEDIT software and a pylogram was constructed using MEGA software. All the 18 samples that were confirmed type 1 by both genotyping PCR found to form a unique clade with B95_8 strain Type 1 standard strain Ag876 (Figure ----). The results of phylogeny correlated with genotyping PCR results signifying the circulation of EBV type 1 in Indian infectious mononucleosis patients

Phylogenetic Analysis of Samples Positive for EBNA2 From Pediatric infectious mononucleosis patients



Phylogenetic Analysis of Samples Positive for EBNA3C from Pediatric infectious mononucleosis patients



The evolutionary history was inferred using the Neighbor-Joining method [1]. The optimal tree with the sum of branch length = 1.89528877 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [2] and are in the units of the number of base substitutions per site. The analysis involved 20 nucleotide sequences. Evolutionary analyses were conducted in MEGA5 [3].

VI. CONCLUSION

To clarify the type of strains prevalence and pathogenesis of EBV it is essential to determine the genotypes, additionally, the examination of sequence diversity among different isolates of the virus is important because the variants may differ in their pattern of reactivation and pathogenesis. EBV genotyping by Phylogenetic analysis based on nucleotide sequence gives the most reliable genotyping results, however, this is inappropriate for large scale assay of genotyping. In order to address this issue we have use a rapid and specific genotyping system using PCR methodology targeting LMP, EBNA2 and EBNA3C genes express in latent state of EBV since EBV DNA show latency in all infections and associated disorders.

Much work which has been done previously was based on EBNA2 and EBNA3C for genotyping which classify two subtype of EBV has type1 (type A) which is predominant in

develop and developing countries and type2 (typeB) which is predominant in Africa. In designing the genotype-specific PCR primers consideration has been given to high matching for the entire sequences and also matching of the two or three nucleotide at 3' end which is one of the key parameters for specific priming. The sequence within the same genotype are different by <2 nucleotides among the entire sequence of the genotype-specific primer while the sequence within the different genotypes has a difference of >3 nucleotides.

CONFLICT OF INTEREST

The authors declare that there was no conflict of interests.

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