RESEARCH PAPER

Genotypic Detection of Epstein-Barr Virus in Pediatric Transplant Recipients From India

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Objective: To determine the rate of occurrence and genotypes of Epstein-Barr Virus (EBV) among pediatric renal and liver transplants recipients.

Design: Observational study.

Setting: Vision Research Foundation referral center and Institute of Liver Disease and Transplantation, Chennai, India.

Participants: 70 pediatric solid organ transplant recipients and 60 voluntary healthy donors.

Methods: Polymerase chain reaction (PCR) for detection and genotyping of EBV were carried out using genes targeting Viral capsid antigen, Nuclear antigen 1, 2 and 3, followed by real time PCR for viral load determination and further confirmed by phylogenetic analysis.

Results: EBV was detected in 35 (51.4%) samples (32 liver and 4 renal transplants) with high viral load. Type A was detected in 33 samples, Type B in 2 liver transplant patients, and co-infection in one liver transplant patient who developed Post-transplant Lymphoproliferative Disorder (PTLD). Real time PCR results correlated with conventional PCR. The mean viral load for patients who did not develop PTLD was 50,424 copies/mL. Overall EBV load in patient with PTLD ranged from 1,40,392 copies/mL prior to PTLD diagnosis to 62,124 copies /mL post treatment.

Conclusion: EBV infection is the high risk factor for PTLD after liver transplantation. PCR targeting of EBV can be applied to diagnose EBV infections and monitor treatment for EBV in pediatric solid organ transplant recipients.

Keywords: *Phylogenetic analysis, Polymerase chain reaction, Post-transplant lymphoproliferative disorder.*

Epstein-Barr virus (EBV) is recognized as a primary pathogen causing Infectious mononucleosis [1]. Monitoring of EBV DNA in peripheral blood is routinely performed in transplant centers because these patients are at higher risk to develop EBV-associated diseases including the potentially life-threatening post-transplant lympho-proliferative disorder (PTLD). In most cases, PTLD is associated with EBV infection of B cells, either as a consequence of reactivation of the virus post transplantation [2,3] and intensity and type of immune suppression [4]. The incidence of PTLD reflects the more intensive use of immunosuppressive drugs, possibly in combination with the varied EBV load in the transplanted organ [5]. PTLD is most likely caused by iatrogenic suppression of T-cell activity in transplantation recipients, which leads to inadequate immune surveillance against EBV-induced proliferation of infected B-cells [6]. Currently there is no definitive treatment regimen for PTLD prevention [7].

EBV is divided into two subtypes, type A and type B that are distinguished by genomic difference in a subset of latent genes that encode for the EBV nuclear antigens 2 (EBNA2) [8], EBNA 3A, 3B and 3C [9]. EBV is frequently detected in blood samples from healthy individuals, usually EBV type A [9], while immuno-suppressed individuals (HIV-infected and transplant patients) have a high rate of infection with EBV type B. Though EBV has been associated with PTLD, only a few detailed studies involving pediatric patients have been carried out, and none from India. Studies of EBV infections are limited due to the lack of routine culture techniques, and poor reliability of serology [10]. PCR is an attractive diagnostic tool in this setting because of its sensitivity. The detection and quantification of EBV-DNA load in peripheral blood has been utilized as a prognostic marker for the development of PTLD. This study aims at determining the presence of EBV in pediatric transplant recipients and to know the most common genotype present among them.

METHODS

The study was approved by the institute's ethics sub-committee. Informed consent was obtained from the patient's kin. Clinical details were recorded in the proforma made specifically for the study. Samples of peripheral blood (2-3 mL) were collected in plain and EDTA-coated vacutainers. Samples were processed immediately for serological analysis. Samples for molecular detection were stored at minus 80°C.

Serological investigation of patients prior to transplantation and healthy controls consisted of anti-viral capsid antigen (VCA) immunoglobulin M(IgM) and anti-VCA IgG, anti-Epstein–Barr Nuclear antigen (EBNA) IgM, and anti-EBA IgG testing using enzyme-linked immunosorbent assays (ELISA) with recombinant antigens following instructions of the manufactures (Demeditec Diagnostics, Germany). A positive result for anti-VCA IgG was defined as EBV seropositive. Patients who had detectable IgM antibodies to VCA and absence of VCA-IgG were considered to have early primary infection. Recent infection or reactivation was defined as a positive assay for both IgM and IgG to VCA, and a negative assay for both IgM VCA and IgG VCA were defined as no EBV infection. Serological investigations for other infectious agents like Cytomegalo Virus (CMV), Herpes Simplex Virus (HSV) 1 and HSV 2 was also performed. All the samples were subjected to viral load determination by real time PCR and genotyping by type specific PCR. PTLD diagnosis was based on clinical and histological criteria.

The standard immunosuppressive regimen consisted of tacrolimus (fujimycin) with or without mycophenolate mofetil. Target tacrolimus trough levels in plasma were as follows: 12-15 ng/ mL for the first 2 weeks after transplant, 10 ng/mL for the second through fourth weeks, 5-8 ng/mL for the first through sixth months, 5 ng/ mL for the sixth through 12th months, and 2-3 ng/mL after the 12th month. When a liver or renal transplant recipient who was positive for EBV developed clinical symptoms or the blood EBV load detected; immuno-suppression with tacrolimus was gradually decreased and kept at the minimum considered safe. Oral acyclovir (30-60 mg/kg/day) was administered until the EBV load decreased. No patient received antiviral prophylaxis in this study.

Samples of peripheral blood (2-3 mL) were collected in plain and EDTA coated vacutainers from voluntary healthy donors (n=60). Age group of the control group ranged between 17-20 years. EBV Standard Strain Type A: Culture infiltrate of Marmoset cell line infected with EBV B958 (National Eye Institute, Bethesda, USA), EBV Standard Strain Type B: Culture infiltrate of Ag876 cell line (*Source: Dr Alan Rickinson, Glasgow University, Germany*). DNA was extracted from all samples following the manufacturer's instructions of QIAGEN DNA extraction kit, Hilden, Germany.

In order to confirm the presence of EBV, two PCRs targeting the genes that code for EBV-VCA and EBNA1 were standardized and applied to all samples. All the PCRs were optimized to be carried out in the same thermal profile. 50µL of the PCR mix contained 10 µL of extracted DNA, 100mM of each dNTP, 5 µL of 10× PCR buffer, 1 µM of each forward and reverse primer and 3U/µL Taq DNA polymerase. PCR was carried out denaturing the DNA at 94ºC for 5 minutes followed by amplification for 30 cycles, by secondary denaturation at 94ºC for 1 minute, annealing at 59ºC for 1 minute and extension at 72ºC for 1 minute with final extension for 7 minutes at 72ºC. For the second round of amplification 5µL of the first round product was added to 45µL of the PCR mix containing 10 mM of each dNTP, 10× buffer, 1 µM of each forward and reverse primer and 3 U/µL Taq DNA polymerase. The PCR amplification was carried out for 20 cycles with the same thermal profile as mentioned above. Two controls (a reagent control and a 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 amplified positive controls. To prevent contamination, DNA 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 5µg mL^{-1} of ethidium bromide in 1×Tris-Borate buffer (pH -8.2-8.6) and documented on gel documentation system (Vilber Lourmat, France). The viral load was estimated in the DNA extracts of all test and control samples using a commercial kit - RoboGene Quantification Kit (Hilden, Germany). The assay was performed on Rotor Gene (Hilden, Germany) real time PCR equipment. The amplification reaction was carried out following the manufacturer's instructions. PCR was carried out at 50ºC for 30 minutes followed by initial denaturation at 95ºC for 15 minutes followed by 50 cycles of initial denaturation at 95°C for 30 seconds, annealing at 50ºC for 60 seconds and extension at72ºC for 30 seconds. The viral load was expressed as copies/ mL. The samples that were found positive for EBV were subjected to genotyping by PCR targeting the EBNA2 and EBNA3C genes. Uniplex PCR for detection of EBNA2, EBNA3C genes was standardized using the EBV-A and EBV-B Standard Strains. Primers targeting

genes that codes for EBNA2 and EBNA3C genes were designed using Primer premier Biosoft international, USA, based on consensus sequence 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* I**. All primers and PCR reagents were procured from VBC – Biotech service, Vienna. The PCR positive -amplified products were further subjected to DNA sequencing and compared with the standard strain sequence to determine the homology percentage. Cycle sequencing of the amplified products was performed in a 10µL reaction volume, containing 0.5µL of RR mix, 3.5µL of sequencing buffer, 1µL of forward primer (1:100 diluted), 1µL of reverse primer (1:100 diluted)2 iL MilliQ water, and 2µL of amplified product. Amplification was carried out in the Perkin- Elmer thermocycler using 25 cycles at 96°C for 10 s, at 50°C for 5s, and at 60ºC 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 analyzed by Bio Edit sequence alignment software, (www.softpedia.com/progDownload/BioEdit-Download-174716). BLAST analysis (www.ncbi.nlm.nih.gov/BLAST) was done to compare and confirm the sequenced data with the standard strains and to determine the homology percentage. The nucleotide sequences of the EBNA2 and EBNA3C PCR positive amplified products were analyzed by comparison with EBV standard strain nucleotide sequences using BIOEDIT software. Evolutionary distances were estimated by constructing a phylogram using UPGMA algorithm by performing bootstrap analysis (Replicates 100) in CLC Main Workbench6.71 software. The statistical significance of PCR on diagnosis of EBV in transplant patients was done using Fisher's exact test. Mean, Standard deviation, median and Box plot for viral load were determined using SPSS14.

RESULTS

Peripheral blood samples were obtained from 70 pediatric solid organ transplant recipients; 24 were renal and 46 were liver transplant recipients. The most common clinical conditions presented were acute renal failure, Hepatitis, encephalitis, interstitial nephritis and Glomerulo-nephritis. Eight of the 70 patients were positive for IgG VCA. Two of the 70 patients were positive for IgM VCA and seven of the 70 patients were positive for IgG EBNA VCA. None of the 70 patients were positive for IgM EBNA. Serological tests for detection of other viruses showed IgM CMV in five, and IgM and IgG to CMV in six patients. Seven patients were positive for IgG HSV1 and one of the patients was positive for IgM HSV1. None of the controls tested positive to IgM VCA, whereas nine samples tested positive to IgG VCA.

Thirty-five samples (50%) tested positive for both VCA and EBNA1. Eight (13.3%) control samples tested positive for EBNA1 PCR. None of the controls tested positive f22or EBV VCA and no detectable copy numbers were found by Real time PCR. All the test samples that tested positive by nPCR were also tested positive by real-time PCR. The mean viral load for EBV PCR positive patients who did not develop PTLD was 50,424 copies/mL (Lowest Viral Load: 14 copies/mL and

TABLE I LIST OF PRIMERS USED FOR AMPLIFICATION OF GENES THAT CODE FOR VCA, EBNA1, EBNA2 AND EBNA3C OF EBV

Gene	*Primer*	*Primer sequence*	*Expected Base Pair*
VCA	EBV F I	5'-TTTGGCGTCTCAGGCTAT-3'	Round 1: 172 Round 2: 126
	EBV PP R	5'-CGTGGTCGTGTTCCCTCA-3'	
	EBV PP F	5'-CGGTGTAACTACCCGCAATG-3'	
	EBV PP R	5'-CGTGGTCGTGTTCCCTCA-3'	
EBNA1	EBV up	5'-GCAGTAACAGGTAATCTCTGG-3'	Round 1: 490 Round 2: 336
	EBV low	5'-ACCAGAAATAGCTGCAGGACC-3'	
	EBV up (R)	5'-GATTTGGACCCGAAATCTGA-3'	
	EBV low (R)	5'-CCTCCCTAGAACTGACAATTGG-3'	
EBNA2	EBNA-2 F	5'-TTTCACCAATACATGAACC-3'	Type A: 378 Type B:483
	EBNA-2R	5'-TGGCAAAGTGCTGAGAGCAA-3'	
EBNA3C	EBNA3C-F	5'-AGAAGGGGAGCGTGTGTTGT-3'	Type A: 153 Type B: 246
	EBNA3C-R	5'-GGCTCGTTTTTGACGTCGGC-3'	

Highest Viral Load: 8,20,955 copies/mL). One of the 70 post-transplant patients developed PTLD four months post-transplant. The patient had CMV infection acquired 3-6 weeks post liver transplant which was successfully treated with gancyclovir. The PTLD coincided with strongly increased levels of EBV DNA load in the peripheral blood. The highest titer value of 11,63,900 copies/mL was detected in the blood collected from this PTLD patient. Real-time PCR EBV titre from lymphoid biopsy of this patient was 89,14,188 copies/mL. The viral load for this patient prior diagnosis of PTLD was 11,63,900 copies//mL, which was significantly higher compared to the load in the liver transplant recipients who did not develop PTLD. The clinical presentations of EBV positive pediatric renal and liver transplant recipients are given in ***Web Table* I**.

Genotyping of type A and type B was done by targeting EBNA2, EBNA3C genes. Type A was detected in thirty two (45.7%) and type B in blood of two (2.9%) samples (***Table* II**). The blood and lymphoid tissue of the patient who developed PTLD revealed mixed subtypes, a co-infection with both A and B EBV genotypes. Both samples that tested positive for EBV Type-B genotype were found to have higher titre values than all of the EBV type-A positive samples (1,23,714 copies /mL and 8,20,955 copies/mL). Eight control sample tested EBNA1 PCR positive revealed prevalence of EBV Type A genotype by both genotyping PCRs **(*Table* II**). All PCR positive samples were subjected to sequencing. Sequencing of the samples re-confirmed the PCR results. The full length sequences were submitted to Genbank database and the assigned accession numbers are KC884748 – KC884757 and KF429681 – KF429706. Comparison of EBNA2 and EBNA3C sequences with EBV standard strain sequence, showed type A to form a unique clade with B95_8 strain and type B to form a separate clade with EBV Type B standard strain Ag876.

DISCUSSION

In this study of 70 solid organ transplant recipients, we found EBV type A to be more prevalent in pediatric transplant patients as compared to EBV type B. Samples positive for EBV type B had significantly higher titre values than Type A samples. The sample with co-infection had the highest titer values and this patient also developed PTLD. All the patients except the patient who developed PTLD responded to the drugs and recovered from EBV illness (EBV titer reduced).

The limitations in our current study were the follow-up samples were not collected or diagnosed for all the patients. The immunological response during active EBV infection was not detected. Knowledge about the pathogenic factors of PTLD may help in the development prognostic markers and therapeutic strategies for treating EBV induced PTLD in immunocompromised post-transplant patients.

Every center should have a high-risk group which would include patients based on previous studies and the centers own experience. Factors for high-risk patients could include EBV sero-negativity at the time of transplant, active primary EBV infection at the time of transplant, underlying disease leading to transplantation, prior splenectomy, second transplant, patient age (children and older adults), co-infection by cytomegalovirus and other viruses, acute or chronic graft-versus-host disease, immunosuppressive drug regimen and intensity, cytokine polymorphisms, HLA type and extent of HLA mismatch, and the presence of multiple risk factors on this list [12]. Levels often rise before clinical diagnosis of PTLD, allowing pre-emptive intervention in high-risk patients who are routinely monitored for EBV levels [12]. In our study, the viral load was higher in the patient who developed PTLD before the diagnosis was made compared to the samples received from the patient after diagnosis was made and treatment had started. Overall EBV DNA load in this patient decreased from 1, 40,392 copies /mL before diagnosis of PTLD to 1032 copies/mL blood, after diagnosis and treatment and finally EBV negative.

Despite having only one patient who developed PTLD we suggest that EBV viral load could act as a good diagnostic tool to improve prediction of PTLD in transplant patients. Developing better PTLD prediction tools using high and low risk patient groups will surely improve patient treatment. Patients who fall in the High-risk group can be focused on for regular follow-up and treatment.

TABLE II VIRAL LOAD IN EBV-POSITIVE PATIENTS

Genotype	*No. of samples*	*Mean viral load*	*Median viral load*	*Standard deviation*
EBV Type A	32	24055	1532	46970
EBV Type B	2	472335	472335	493024
EBV Type A & B	1	1163900	1163900	N/A

WHAT IS ALREADY KNOWN?

- No studies are available on prevalence of EBV among pediatric post-transplant patients from India.

WHAT THIS STUDY ADDS?

- Type A EBV was the most prevalent EBV subtype in pediatric transplant cases.

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