A Clinico - Molecular Biological and Genotypic Study on Epstein -Barr Virus in Chennai Population

THESIS

Submitted in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

by

M. K. JANANI

Under the Supervision of

Dr. J. MALATHI

&

Under the co-supervision of

PROF. SANJEEV KUMAR

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE

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CERTIFICATE

This is to certify that the thesis entitled **"A Clinico - Molecular Biological And Genotypic Study On Epstein -Barr Virus In Chennai Population"** and submitted by **MS. M. K. JANANI** ID No **2010PHXF041P** for award of Ph. D. Degree of the Institute embodies original work done by her under my supervision.

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ABSTRACT

Epstein-Barr virus (EBV) is recognized as a primary pathogen causing infectious mononucleosis, a usually benign lymphoproliferative disorder most prevalent in adolescents and young adults. Studies of EBV infections are limited due to the lack of routine culture techniques. Currently, laboratory diagnosis of primary or reactivated Epstein - Barr virus infection depends on serologic tests which are an unreliable diagnostic tool in case of immunocompromised patients where patients show a marked delay in their humoral response to EBV antigens, and many fail to produce immunoglobulin (IgM) antibodies altogether. EBV infection can be mistaken for Human Cytomegalo virus (HCMV) infection or streptococcal, throat infection or an ordinary fever, strictly because of the symptoms overlap. False-positive IgM reactions occur due to auto antibodies or other serum factors and due to anamnestic reactions or cross-reactions to other recent infections. Antigenic cross-reactivity among the two herpes viruses, Epstein – Barr virus and Human cytomegalo virus usually account for false-positive serological results. IgM antibodies induced during EBV infection cross react with the N-terminal half of Epstein – Barr Nuclear Antigen as well as with pUL44 and pUL57 proteins of HCMV. Differentiation of acute from chronic and differential diagnosis of EBV induced Infectious mononucleosis (IM) from Infectious Mononucleosis like syndrome caused by other infectious agents is the key area of diagnosis. Hence, there is need for an alternative detection method which is specific, sensitive and rapid, particularly where serological proof of diagnosis is lacking. Therefore, in this study Polymerase chain reaction (PCR) targeting Epstein-Barr viral capsid antigen (VCA) was standardized and evaluated against Enzyme linked Immunosorbent assay. Thus, PCR targeting Viral Capsid Antigen standardized in this study will be a rapid, reliable and potentially serve as a diagnostic marker for the early diagnosis of Epstein - Barr virus.

EBV is divided into two subtypes, type A and type B, distinguished by genomic difference in a subset of latent genes that encode for the EBV nuclear antigens (EBNA) such as EBNA2 and EBNA 3A, 3B and 3C. EBV is frequently detected in blood samples from healthy individuals, and most EBV serum-positive healthy Caucasians are infected with EBV type A, while immunosuppressed individuals (HIV-infected and transplant patients) have a high rate of infection with EBV type B. EBV infection is the high risk factor for Post transplantLymphoproliferative Disorder (PTLD) after liver transplantation. The quantification of Epstein – Barr viral load in the peripheral blood have been utilized as the prognostic marker for the diagnosis of EBV induced Post transplant lymphoproliferative disorder. Establishing the presence of EBV would have a definite impact on understanding of the spectrum of EBV disease, pathogenesis, and management of the increasingly common EBV related lymphoproliferative disorders. Understanding the frequency of the virus in a population and identifying the various types present would shed light on the biology of these viruses and improve our understanding to monitor them. Studies on detection and genotyping of EBV among pediatric transplantation patients who have undergone solid organ transplantation are limited. EBV infection is the high risk factor for PTLD after liver transplantation. Therefore the second part of the study aims at determining the presence of EBV load in pediatric transplant patients and to know the most common genotype present among them and anti-viral cytokine expression was analyzed to understand the pathophysiology of EBV-associated infection. Real time PCR performed for determination of viral load and the anti-viral cytokines expression in response to EBV infection in post liver transplantation patients revealed significantly higher levels of viral copy numbers in liver transplant recipients than patients with EBV induced Infectious Mononucleosis like syndrome. High levels of IL-2, IL-6, IL-1B, IL-17A and IL-10 were observed in a patient with post transplantlymphoproliferative disorder. Genotyping studies performed using two gene targets of EBV, EBNA2 and EBNA3c, revealed prevalence of Type A in majority and circulation of both genotypes in a patient who developed PTLD.

EBV is also associated with various ocular diseases like keratitis, uveitis and in some rare cases Acute Retinal necrosis Syndrome (ARNS) leading to retinal inflammation and visual loss. Though many reports are available on detection of EBV, but there is no information available about the EBV genotype associated with the ocular infections. Therefore the next part of the study was designed to determine the rate of occurrence of EBV in ocular infections and also to find the genotypic prevalence of EBV. Genotyping studies revealed the prevalence of only EBV subtype A in all test and control ocular samples. Since EBV type A was found to be prevalent in causing ocular infections, further study was designed to determine whether EBV Type A strain can infect Adult Retinal Pigment Epithelial cells (ARPE-19) *in-vitro* and the replication of virus is involved in the inflammation of retinal epithelial cells. The inflammatory potential of EBV recombinant proteins (p23 and nuclear antigen) in Adult Retinal Pigment Epithelial cells (ARPE-19) was determined. EBV Type A strain and Epstein – Barr viral recombinant protein exposure lead to the pro inflammatory cytokines release by ARPE-19 cells. Toll like Receptors (TLR) were involved in the innate immune response via MyD88 signaling. EBV type A strain and EBV recombinant proteins induced TLR7 and TLR8 expression in the transcript level and this was followed by nitric oxide (NO) production via iNOS. As a sum of these stress responses the cells underwent apoptosis which ultimately lead to cell death in ARPE-19 cells. In conclusion both p23 and Nuclear Antigen mediated inflammatory response in ARPE-19 cells. This is the first *in vitro* study to elicit the Novel Immune Regulatory Pathway Associated with Epstein - Barr virus and the Recombinant Epstein – Barr Viral Protein Mediated inflammation in Retinal Pigment Epithelium (ARPE-19). These findings provides crucial information for understanding the immune mechanisms of EBV induced inflammation and cell death in ARPE-19 and help to design new immunotherapeuticals approaches to treat ocular infections caused by EBV.

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CHAPTER 1

1. Introduction:

Epstein-Barr virus (EBV) is an extraordinary successful virus, infecting almost every adult human being on the planet. Since its discovery, it has been increasingly implicated in the pathogenesis of a wide spectrum of lymphoid and epithelial malignancies.

1. 1. History:

EBV was discovered by Epstein, Achong, and Barr (Epstein et al. 1964). It was originally discerned as virus-like particles by electron microscopy in the cells of biopsied samples from Burkitt's lymphoma (BL) (Epstein et al. 1964). Since its identification, scientific research has led to the association of EBV with infection and malignancies in humans. Thus, EBV is identified as the first candidate human tumor virus.

1. 2. Infection:

Primary infection of Epstein-Barr virus (EBV), which is primarily transmitted by saliva, actively replicates in the epithelial cells of the oropharynx and can subsequently infect recirculating B lymphocytes which may lead to acute infectious mononucleosis (glandular fever). Infectious mononucleosis is a benign lymphoproliferative disease that is usually seen in children, although most cases of EBV infection occur in early childhood and have no symptoms. It is characterized by transient immunosuppression and an unusual expansion of atypical lymphocytes, the majority of which are not B cells but $CD8^+$ T cells. Instead, in these cells EBV establishes a latent infection that persists for life during which only a few viral genes are expressed. Latent infection is a common feature to other herpesviruses (Cayrol and Flemington, 1995; Cooper, 1994). EBV has a narrow tissue tropism limited to B lymphocytes, T lymphocytes and epithelial cells of primate origin. By the age of twenty, more than 90% of humans are seropositive, demonstrating previous exposure to EBV.

EBV is potentially oncogenic and has been linked to Burkitt's lymphoma, nasopharyngeal carcinoma, B cell lymphoma, X-linked lymphoproliferative disorder (Miller, 1990; Jabs, 1996), and more recently to other neoplasia, including Hodgkin's

disease, peripheral T cell tumors, and gastric cancer (Yoshiyama et al. 1995). EBV is also a significant problem in AIDS patients where it is associated with diffuse polyclonal lymphomas, lymphocytic interstitial pneumonitis and oral hairy leukoplakia of the tongue. EBV positive Burkitt's lymphomas are observed with increasing frequency in transplant recipients receiving immunosuppressive therapy. In normal individuals, latent EBV infection is controlled by humoral immunity, cytotoxic T cells, and the interferon (IFN) system (Jabs, 1996).

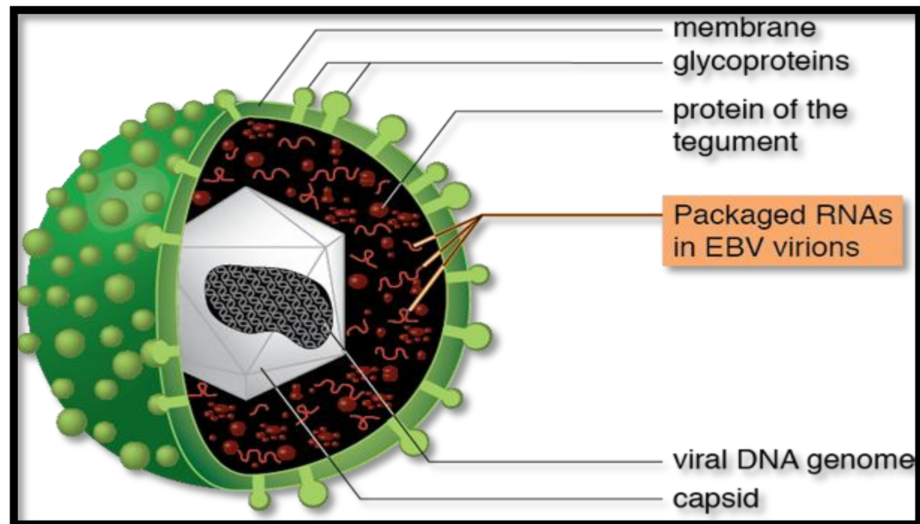
1. 3. Taxonomy:

Epstein - Barr Virus is grouped as a member of the Herpesviridae family, subfamily gammmaherpesvirinae, genus lymphocryptovirus. The Herpesviridae family contain viruses grouped together based on the architecture of their virion.

A typical herpesvirion consists of a core containing a linear, double stranded DNA; an icosahedral capsid, approximately 100-110 nm in diameter, containing 162 capsomeres with a hole running down the long axis; an amorphous, sometimes asymmetric material that surrounds the capsid, designated as the tegument; and an envelope containing viral glycoprotein spikes on its surface. (Roizman, 1990)

1.4. Genome:

The EBV genome isolated from virus particles is a linear, double stranded DNA molecule of about 175 kilo base pairs (kbp). It is characterized by a number of different repetitions. The termini consist of tandem repeats of approximately 540 base pairs (bp). A variable number of large internal repeats of about 3.1 kbp join short and long unique regions. Several different other repeats are interspersed in the genome (Figure 1. 1). Two clusters of small tandem repeats of 125 and 102 bp show partial homology and have the same orientation on the genome. Each cluster is flanked by a highly conserved region of about 1 kbp. (Keiff and Leibowitz, 1990; Miller, 1990). The entire genome persists in the proliferating lymphocytes as linear-integrated and covalently closed circular episomal DNA (Fennewald et al. 1984). The EBV genome has been mapped using Bam HI restriction endonuclease to produce fragments which have subsequently been denoted with a letter to identify specific fragments (Sample et al., 1984).



Courtesy: www.helmholtz-muenchen.de

Figure 1. 1: Structure of Epstein Barr Virus

1.5. Structure of Epstein Barr Virus:

1.5.1. Capsid:

Epstein Barr Virus consist of icosahedral capsid, approximately 100-110 nm in diameter, containing 162 capsomeres with a hole running down the long axis (Sample et al., 1984).

1.5.2. Tegument:

The term tegument, which is common to all herpesviruses, was introduced to describe the structures between the capsid and envelope. These structures have no distinctive features in thin sections, but they may appear to be fibrous on negative staining. (Roizman, 1990).

1.5.3. Envelope:

The most abundant EBV envelope proteins are gp350 and gp220 (Figure 1. 2). Additional viral envelope proteins expressed include gp85, gp25, gp42/38, gp43, and gp78/55 (Cooper, 1994).

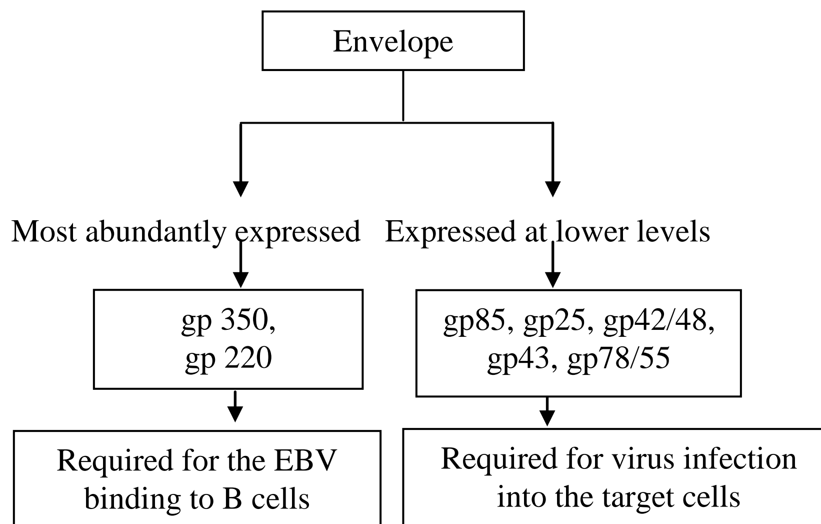


Figure1. 2: Classification of Epstein Barr virus Envelope proteins.

1.5.5. EBV proteins: The proteins characterized into two groups (Cooper, 1994):

A. Latent proteins

1. Epstein Barr viral nuclear antigen complex (EBNA):

EBNA is now known to be a complex of at least 6 proteins, EBNA 1-6. EBNA 1 is thought to be essential for the maintenance of the episomal state of EBV in infected cells and binds to the origin of replication. EBNA 1 is expressed in all known virus carrying cells, but its expression may be lost when a lytic cycle ensues. EBNA 2 has two phenotypes, A and B. Strain A, which is found mainly in Caucasians, is more efficient in immortalizing B lymphocytes than strain B, which is found in Africa.

2. Latent membrane protein (LMP):

LMP is a membrane associated protein which is found in the virus particle and infected cells. The structure of LMP resembles a growth factor receptor which may play a role in the immortalization.

3. Terminal protein:

The terminal proteins are only expressed when the viral genome is circularized as its reading frame spans the terminal repeat sequences.

4. **Lymphocyte-detected membrane antigen (LYDMA):**

LYDMA is the name given to viral coded proteins in expressed cells which are recognized by EBV specific Tc cells.

B._Lytic_cycle_proteins

1. Membrane antigen (MA):

MA consists of at least 3 glycoproteins, gp 350, gp 250 and gp85, which are present on the viral envelope and the cell membranes of infected cells. These molecules mediate the attachment of the virus to the cell and are antibodies against MA are virus neutralizing.

1. **Early antigen complex (EA):**

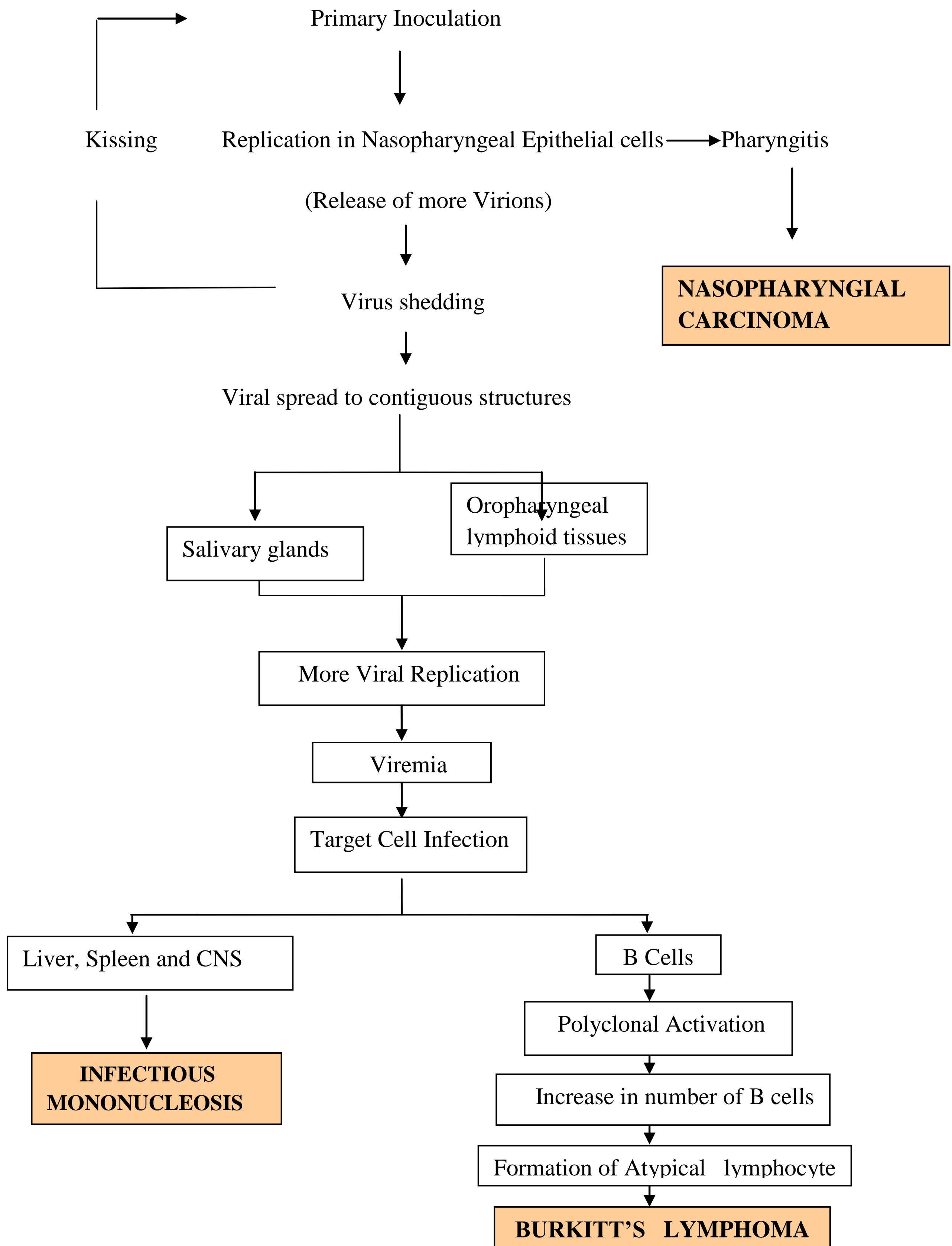
EA is a complex which is only expressed in infected cells undergoing the lytic cycle. The functions of the proteins of the EA complex are as yet unknown.

2. **Viral capsid antigen complex (VCA):**

VCA complex comprises of the structural proteins which make up the viral capsid and are synthesized late in the lytic cycle.

1.6: Transmission Cycle:

Epstein-Barr virus is present in oropharyngeal secretions and is most commonly transmitted through saliva. After initial inoculation, the virus replicates in nasopharyngeal epithelial cells. Cell lysis is associated with a release of virions, with viral spread to contiguous structures, including salivary glands and oropharyngeal lymphoid tissues. Further viral replication results in viremia, with subsequent infection of the lymphoreticular system, including the liver, spleen, and B lymphocytes in blood (Figure 1. 3).


EBV- Transmission through Saliva
Primary Inoculation
Kissing
Replication in Nasopharyngeal Epithelial cells
Pharyngitis
(Release of more Virions)
Virus shedding
NASOPHARYNGIAL CARCINOMA
Viral spread to contiguous structures
Salivary glands
Oropharyngeal lymphoid tissues
More Viral Replication
Viremia
Target Cell Infection
Liver, Spleen and CNS
B Cells
INFECTIOUS MONONUCLEOSIS
Polyclonal Activation
Increase in number of B cells
Formation of Atypical lymphocyte
BURKITT'S LYMPHOMA

1.7 **Figure 1. 3:** Transmission cycle of Epstein Barr Virus

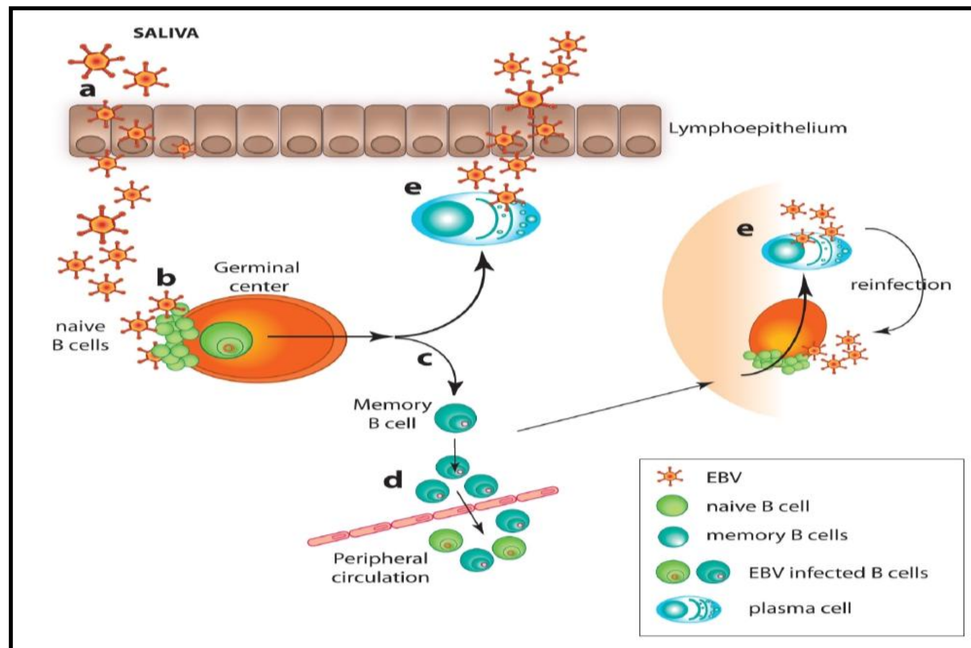


Figure 1.4: Hypothetical model of EBV infection and persistence. **a** During primary infection EBV enters through saliva into the crypts of lymphoepithelial structures such as the tonsils. EBV crosses the epithelial barrier. **b** infects the naïve B cells that are in the resting state in the lymphoid tissue of Waldeyer's ring and activate these cells to become proliferating blasts. **c** The antigen-activated B cell-blast is rescued through entry into the pool of memory B cells when it receives signals from antigen and antigen-specific helper T cells. Memory B cells occasionally divide, as part of the homeostatic mechanism for maintaining stable numbers of cells. **d** EBV latently infected resting memory cells leave the follicles and enter the peripheral circulation, from where they re-enter the tonsil. **e** In response to unknown signals (perhaps polyclonal activators and/or bystander T cell help), memory cells may differentiate into plasma cells and secrete antibody. This differentiation may be related to the mechanisms that sustain lifetime production of antibody. If such cell contains the virus, it will reactivate viral replication and infectious virus will be produced (Straus 1993).

1.8: Lytic Infection:

Lytic infection occurs early after primary inoculation. As a result of lytic infection in oral epithelial cells, Epstein-Barr virus can be found in the saliva for the first 12-18 months after acquisition. Thereafter, epithelial cells and lymphocytes are latently infected, with a few spontaneously converting, leading to viral replication, host cell lysis and death, and release of mature virions. Thus, the virus can be isolated from

oral secretions of 20-30% of healthy latently infected individuals at any time (Cooper, 1994).

1.9:Latent Infection:

During latent infection, cell proteins are expressed in 1 of 3 patterns. Type I latency, associated with Burkitt lymphoma, is characterized by expression of only Epstein-Barr virus–encoded RNAs, Epstein-Barr early regions (EBERs), and Epstein-Barr nuclear antigen 1 (EBNA1). Type II latency, associated with nasopharyngeal carcinoma, is characterized by expression of 3 latent membrane proteins (LMP1, LMP2A, LMP2B), plus EBERs and EBNA1. Type III latency is the pattern found in healthy individuals with latent infection. In addition to the EBERs and EBNA1 expressed in type I latency, other nuclear antigens (including EBNA2, EBNA3A, EBNA3B, EBNA3C, and LMP) are expressed in type III latency (Miller, G. 1990).

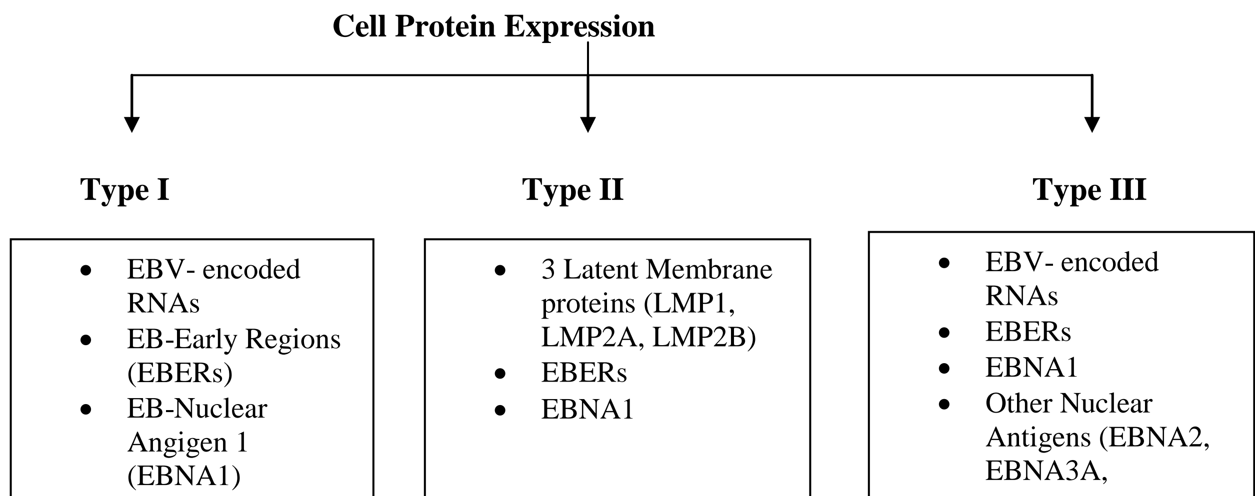


Figure 1.5: Latent EBV protein expression

1.10. Malignancies Associated with EBV:

A variety of malignancies has been linked to EBV; such as more than 95% of the Endemic Burkitt's lymphoma (BL) (Magrath 1990), all of the PTLD, part of Hodgkin’s lymphoma and certain types of T-cell lymphoma (Jones et al. 1988). The virus is also present in all undifferentiated nasopharyngeal carcinoma (NPC) (Raab-Traub and Flynn 1986) and 10% of the stomach cancers . Besides, the virus is also found to be associated with a number of other rarer cancers, such as nasal T-

cell/natural-killer lymphomas, lymphomatoid granulomtosis, angioimmunoblastic lymphadenopathy, and central nervous system lymphomas in immunocompromised patients (Harabuchi et al. 1990).

1.11.Diagnosis of EBV:

1. 11. 1: Complete blood count:

Leukocytosis with a WBC count of 10,000-20,000 cells/ml is found in 40-70% of patients with acute infectious mononucleosis. By the second week of illness, approximately 10% of patients have a WBC count more than 25,000 cells/ml.

Approximately 80-90% of patients have lymphocytosis with more than 50% lymphocytes. Usually, 20-40% of the lymphocytes are atypical, although not all patients have more than 10% atypical lymphocytes. The atypical lymphocytes of Downey types are larger, have a lower nuclear-to-cytoplasmic ratio, and have a nucleus that is less dense than that of normal lymphocytes (Hess 2004).

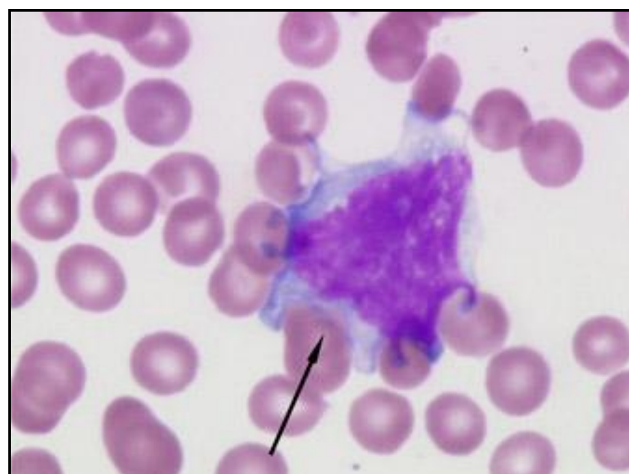


Figure 1. 6: Blood smear showing the presence of atypical lymphocyte: Arrow indicating the presence of “atypical" lymphocytes in the Giemsa stained peripheral blood smear collected from patient suspected with infectious mononucleosis at 1000x magnification. The atypical lymphocytes are larger (more cytoplasm).The cytoplasm tends to be indented by surrounding RBC's.

Most patients with acute infectious mononucleosis have elevated liver function test results. Alkaline phosphatase, aspartate aminotransferase (AST), and bilirubin levels peak 5-14 days after onset, and gamma-glutamyltransferase (GGT) levels peak at 1-3 weeks after onset which determines the levels of these enzymes more sensitively reflect liver-cell damage. Increased AST and GGT activity in infectious

mononucleosis indicates liver involvement and the early elevation of the transaminases was helpful in the early diagnosis of cases with negative serology (VCA-IgM negative) (Hess 2004).

1. 11. 3: Detection of Hetrophile Antibody:

Following infection with Epstein-Barr virus (EBV) in infectious mononucleosis (IM), 85-90% of patients produce specific IM heterophile antibodies. Blood serum in IM often contains an antibody known as heterophile antibody that agglutinates, or clumps, the red blood cells of sheep. Heterophile antibodies are antibodies that are stimulated by one antigen and react with an entirely unrelated surface antigen present on cells from different mammalian species.

Heterophile antibody titers rise during the first two or three weeks with half or more developing a significant titer during the first week of illness. The level of antibody gradually declines and usually disappears in eight to twelve weeks following the onset. Elevated titers sometimes linger for four to six months up to a year or more. Heterophile antibody most commonly used in the serological diagnosis of IM is an IgM antibody which agglutinates sheep red blood cells. These antibodies can be detected by Paul-Bunnell test, Davidsohn differential test and Monospot test (Hess 2004).

1.11.3.1. Paul-Bunnell test:

Sheep red blood cells agglutinate in the presence of heterophile antibodies. It is a usual presumptive test, using unabsorbed serum and 2% suspension of washed sheep RBC. First heat inactivated patient’s serum and 2% sheep RBC are mixed and incubated at room temperature for 2 hours. Patient’s serum containing antibodies due to infectious mononucleosis react with sheep RBC and result in agglutination which indicates positive test for IM (Hess 2004).

1.11.3.2. Davidsohn differential test:

Theoriginal Paul-Bunnell test was a simple titration of sheep cell agglutinins but this procedure was subsequently modified in order to distinguish between sheep cell agglutinins formed in IM and the Forssman-type antibodies found in normal serum,

serum sickness and in certain other conditions. Tissues rich in Forssman antigen (guinea pig kidney) absorb Forssman antibodies but do not affect the heterophil antibodies in IM. Heterophil antibodies are absorbed by beef cells. In this test two types of sheep agglutinins are distinguished by titrating them before and after absorption with guinea pig kidney and ox cells. Patient's serum containing antibodies due to IM is added to guinea pig kidney cells. These antibodies are not absorbed by the kidney cells. These antibodies then react with Beef (Ox) red blood cells which cause agglutination and are a positive test for IM. False positive heterophile antibody tests are rare but may be associated with infection due to Rubella, Cytomegalo virus, Toxoplasmosis, Herpes, Rheumatoid arthritis, Systemic lupus Erythematosus, Leukemia, Lymphomas.The heterophile test is less useful in children younger than 2 years. False negative results are also more likely to occur in elderly patients. Advantages of this test are when it is properly performed this test is specific for Infectious Mononucleosis and false-positive results are rare. The major disadvantage of Davidsohn Differential test is very time consuming and burdensome (Hess 2004).

1.11.3.3. Monospot test:

The latex agglutination assay, which is the basis of the Monospot test using horse RBCs that agglutinates on exposure to heterophile antibodies. Monospot test produce a false-positive result with other disorders. These causes of false-positive Monospot test results include toxoplasmosis, rubella, lymphoma, and certain malignancies, particularly leukemias and/or lymphomas (Hess 2004).

1. 11. 4: Immunoflurescence Assay:

Immunoflurescence staining performed to detect the presence of EBV antigens like viral capsid antigen, nuclear antigen and latent membrane protein in the patients sample and infected culture harvest using antisera targeted against varying EBV antigens (Schmitz and Kampa, 1979, Sener et al. 2009).By this procedure EBV-specific antibodies bind to EBV infected target cell and complement-fixing antibodies against primary antibodies are stained by adding complement and, subsequently, anticomplement fluorescein conjugate. Thus, the complement-fixing reactions of antibodies are based on the detection of the EBV-specific immunoglobulin to EBV antigens.

1. 11. 5: Western Blotting:

Western blot analysis provides the advantage of detecting EBV-specific antibodies to multiple EBV-specific antigens simultaneously, and this makes the results of stage-specific diagnostic assays comfortable to interpret and justifies the use of this technology as a confirmation method. VCA antigen p19 is considered a marker that substitutes for the lack of EBNA-1 IgG, since p19 IgG is mostly produced late in the course of infection.

Five different antibodies in patient's sample can be detected using this technique: VCA gp 125 (EBV glycoprotein 125 which is essential for binding of virus to target cell), EA-D (diffused early antigen which is expressed in early infection), EBNA1 (EBV nuclear antigen 1 which is expressed in later stage/ past infection and essential to maintain latency) and recombinant antigens, such as p19 (VCA), p22 (VCA) can be detected.

1. 11. 6: Enzyme Linked Immuno Sorbent Assay:

Enzyme immunoassays are performed for the detection of IgG and IgM antibodies against early antigen (EA), virus capsid antigen (VCA), and Epstein-Barr Nuclear Antigen (EBNA) (De Paschale and Clerici, 2012)**.**. Epstein Barr Virus ELISA Kit is a solid-phase immunoanalytical test. The polystyrene strips are coated with recombinant antigen that bears immunodominant epitopes of EBV antigens. The anti EBV antibodies, if present in the tested sera (plasma), bind to the immobilized antigens and the antibodies being in complexes with antigen are later on recognised by animal anti-human IgG antibodies labelled with horseradish peroxidase. The labeled antibodies are revealed by an enzymatic reaction with a chromogenic substrate. Negative sera do not react and the mild change in color, if present, may be attributed to the reaction background.

1. 11. 7: EBER (EBV ENCODED RNA) IN *SITU* HYBRIDIZATION:

It is considered the gold standard for detecting and localizing latent EBV in tissue samples (Ambinder and Mann 1994). After all, EBER transcripts are consistently expressed in virtually every EBV-infected tumor, and they are likewise expressed in lymphoid tissues taken from patients with infectious mononucleosis, and in the rare

infected cell representing normal flora in healthy virus carriers. The only EBV-related lesion that lacks EBER is oral hairy leukoplakia, a purely lytic infection of oral epithelial cells (Gilligan et al. 1990).

EBER actually represents two RNA species, EBER1 and EBER2, encoded from two separate but homologous viral genes. EBER transcripts are expressed in latently infected cells at levels approaching a million copies per cell (Yajima et al. 2005). Because EBER transcripts are naturally amplified, they represent a reliable target for detecting and localizing EBV in tissue sections by *in situ* hybridization. EBER *in situ* hybridization can be accomplished on paraffin sections or on cytology preparations. A typical 1-day procedure begins with removal of any paraffin followed by treatment with proteinase K and detergent to enhance probe entry into the nucleus where EBER transcripts are located. Any unbound probe is washed away, and then colorization and counterstaining are performed. Interpretation of EBER stains relies on microscopic visualization of the nuclear EBER signal in latently infected cells. Evaluation of cell type and distribution is helpful in evaluating the clinical significance of the result. The primary advantage of EBER *in situ* hybridization is its ability to localize EBV in the context of cytological and histopathological features of the tissue. EBER hybridizations are used routinely for confirming a diagnosis of EBV-driven posttransplant lymphoproliferative disorder (PTLD) (Chadburn et al. 1995). PTLD is a potentially fatal complication of allogeneic transplantation that requires prompts diagnosis and therapy. About 95% of all PTLDs are EBV-associated, as shown by EBER expression by tissue-infiltrating lymphocytes and/or immunoblasts.

1.11. 8: LMP1 Immunohistochemistry:

The relative merits of immunohistochemistry *versus* EBER *in situ* hybridization deserve attention. In fact, LMP1 immunostains are nearly as effective as EBER *in situ* hybridization for identifying EBV in PTLD cases, in Hodgkin’s disease, and in infectious mononucleosis (Hummel et al. 1992).

Some important differences are seen in the distribution of EBER *versus* LMP1 expression in tumor samples. In PTLD samples, LMP1 is typically expressed in about 5% of lesional immunoblasts (range, 0–100%). When the same PTLD samples are stained for EBER, it becomes apparent that many more lymphoid cells are EBV-

infected, but only a fraction of those cells co express LMP1. Immunoblasts are often the subtype of lymphocyte that co express LMP1, whereas small lymphocytes are more likely to express EBER alone. Occasional PTLDs lack LMP1 entirely, even though EBER is clearly positive, implying that EBER is a more reliable target than is LMP1. Nevertheless, LMP1 immunostains are economical and rapid; therefore, they retain a role in clinical evaluation of suspected PTLD cases.

1. 11. 9: Measuring EBV Gene Expression By Immunohistochemistry:

Detection of viral proteins can be achieved by immunohistochemical stains of paraffin sections. Common targets include EBNA1, EBNA2, LMP2A, and BZLF1(Brink et al. 1997a) . Of these, BZLF1, also called ZEBRA, is the only factor that is characteristic of lytic viral replication. In fact, BZLF1 immunostains are quite useful in confirming a diagnosis of oral hairy leukoplakia in tongue biopsies from AIDS patients using commercially available antibody.

1. 11. 10: Southern Blot Analysis of EBV DNA:

Southern blot analysis can be used to determine the clonality of EBV-infected tissues with respect to the structure of EBV DNA (Raab-Traub and Flynn 1986). This assay is based on the presence of variable numbers of terminal repeat sequences at the ends of each EBV DNA molecule. A given cell is apparently infected only once, and each infecting genome contains up to 20 terminal repeat sequences. The relatively unique terminal repeat structure that is present in a given cell is passed along to cellular progeny upon cell division. Oral hairy leukoplakia, representing an infectious process, produces polyclonal viral genomes indicative of lytic viral replication. On the other hand, EBV-associated tumors harbor monoclonal EBV DNA.

To perform the EBV clonality assay, lesional DNA is first subjected to digestion by*Bam*HI restriction enzyme, which cuts at sequences flanking the region where the terminal repeats are located. After electrophoresis and transfer, a labeled internal probe is applied to detect the fragment(s) containing the terminal repeats. Analysis of the band pattern distinguishes monoclonal from oligoclonal, polyclonal, and uninfected tumors, and also reveals whether the sample contains substantial amounts of linear EBV genomes as a consequence of active viral replication.

1. 11. 11: Amplification of EBV DNA:

Amplification methods have been used by many clinical laboratories for detecting EBV in blood, body fluid, or tissue samples. Most remarkably, amplification of EBV DNA from the cerebrospinal fluid of AIDS patients is nearly always indicative of a brain lymphoma, leading oncologists to proceed with lymphoma treatment without the need for brain biopsy. After treatment, disappearance of EBV DNA from the cerebrospinal fluid is associated with better outcomes (Shibata et al. 1991).

From a technical standpoint, PCR amplification of EBV DNA is accomplished using primers spanning conserved EBV sequences, whereas strain typing relies on amplification of polymorphic regions of the viral genome. The inability to distinguish EBV disease from background infection led many laboratory scientists to abandon PCR in favor of EBER *in situ* hybridization for the reliable detection of lesion-associated EBV in biopsy specimens. Indeed, EBER studies remain a mainstay of diagnostic surgical pathology. But improvements in quantitative amplification technology are stimulating a resurgence of interest in amplification strategies for detecting EBV in patient samples.

1. 11. 12: EBV viral load measurement by quantitative DNA amplification:

EBV viral load testing involves quantitative measurement of EBV DNA in patient samples. A typical viral load assay employs PCR to amplify both EBV DNA and a spiked control sequence in nucleic acid extracted from blood samples. The amount of amplification product measured either at the end point of the assay or in real time, can be used to calculate the EBV viral load in copies per milliliter of blood.

The EBV viral load assay has several technical and clinical advantages over other methods of viral detection. First, the test is rapid. Second, it appears that patients with several subsets of EBV-related diseases are massively and systemically infected by EBV, allowing us to screen for these diseases by viral load assays of blood or body fluid, potentially alleviating the need for invasive tissue biopsy. EBV viral load testing appears to be more reliable than serology for evaluating the EBV status of immunocompromised hosts. In fact, recent studies of transplant patients showed that those affected by EBV-driven PTLD have extremely high EBV viral loads, (Bai et al. 1997a).Furthermore, viral load rises as early as several months before the clinical

onset of PTLD, suggesting that the assay might be used to screen high risk populations for purposes of early intervention (Kimura et al. 1999).And finally, EBV viral load decreases on successful therapy, suggesting that the assay should be used to monitor therapeutic efficacy (Macsween and Crawford 2003a)

CHAPTER 2

2. 1. REVIEW OF LITERATURE:

2.1.1. Detection of Epstein - Barr virus in peripheral blood specimens obtained from patients with typical or suspected Infectious Mononucleosis known to be caused by EBV and to correlate the results with the serological and clinical findings.

The clinical features of acute Epstein-Barr virus (EBV) infection overlap those of a variety of other infectious and noninfectious diseases, and reliable laboratory tests are important to aid the differential diagnosis. Although primary EBV infection can be diagnosed by an assay for heterophile antibodies in adults, this assay's sensitivity is low for children (Chan et al. 1998). The detection of immunoglobulin M (IgM) antibody to the virus capsid antigen (VCA) in the absence of antibody to Epstein Barr nuclear antigen (EBNA) is regarded as suggestive of acute primary EBV infection because EBNA antibodies develop only in late convalescence (Lennette E. T.1992). However, false-negative results may occur due to the transient nature of the VCA IgM response. Conversely, false-positive IgM reactions occur due to autoantibodies or other serum factors and due to anamnestic reactions or cross-reactions to other recent infections (Matheson et al. 1990).

The alternative approach of relying on the absence of EBNA antibody in the presence of VCA IgG may also lead to false-positive or false-negative conclusions. In primary infection of children (Chan et al. 2001) or immunocompromised patients, the appearance of EBNA antibodies may be unusually delayed, resulting in a false diagnosis of recent EBV infection (Nystad and Myrmel 2007). Furthermore, passive maternal antibody may confound the diagnosis in infants. Tests of the affinity of IgG antibody have recently been reported to be useful in differentiating recent from past infections (Gray 1995). The discovery of anti-EBV IgM antibodies in a single serum sample, however, does not allow the diagnosis of a current primary infection. IgM antibodies can persist for months after primary infection or reappear during recurrences of EBV infection. The appearance of specific IgM may also be due to a heterotypical immune response caused by intercurrent infections (Möller et al. 1980; Rasmussen et al. 1982; Morgan-Capner et al. 1983). Reactivation of latent viral infection due to transient suppression of cellular immune functions or to polyclonal stimulation during acute Epstein-Barr virus (EBV) infection is well-known

phenomenon (Haukenes et al. 1994; Karner and Bauer 1994) .Antigenic cross-reactivity among the two herpes viruses usually account for false-positive serological results (Balachandran et al. 1987). It is important to differentially diagnose the EBV, HCMV infectious mononucleosis from bacterial infections so as to institute appropriate therapy. The EBV and CMV infectious mononucleosis have to be differentially diagnosed as the duration of treatment varies for the two. During EBV infection, induced IgM antibodies cross react with the N-terminal half of EBNA-1 as well as with pUL44 and pUL57 of HCMV. Serum samples from patients with primary EBV infection frequently scored positive when tested in different HCMV IgM ELISAs, irrespective of whether conventional or recombinant antigens were used for the design of the HCMV IgM assays. Such cross-reactive IgM antibodies were found to be directed against short glycine - rich motifs contained within the nonstructural HCMV proteins pUL44 and pUL57 (Lang et al. 2001). Further analyses revealed that these glycine-rich motifs were major antigenic domains for IgM antibodies induced during HCMV infection. EBV-induced IgM antibodies that reacted with HCMV antigens showed similar kinetics of reactivity in HCMV- or EBV-specific assays in the course of primary EBV infection, indicating that the two populations of antibodies were highly overlapping (Rhodes et al. 1990). Therefore differential diagnosis plays an important role. Hence, the molecular approach for the detection of EBV is very advantageous in the above mentioned context. Since PCR is a target - based amplification method, it is more sensitive.

2.1.2. Detection of EBV on samples collected from pediatric post transplant patients and to study the genotype prevalence of EBV and to correlate the genetic characters (genotypes) with the clinical findings in the study population.

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) (Moss et al. 2001), EBNA 3A, 3B and 3C (Adldinger et al.1992). EBV type A, which is predominantly detected in Asian Nasopharyngeal carcinoma, has a greater potential to transform B lymphocyte than EBV type B (Brooks et al. 1993). EBV is frequently detected in blood samples from healthy individuals, and most EBV serum-positive healthy Caucasians are infected with

EBV type A, while immunosuppressed individuals (HIV-infected and transplant patients) have a high rate of infection with EBV type B (Anon et al. 1996).

Posttransplant lymphoproliferative disease (PTLD) is a severe and frequent complication in allograft recipients, with mortality rates of 50% to 80% (Paya et al. 1999). Main risk factors for PTLD are primary Epstein-Barr virus (EBV) infection(Swinnen et al. 2000, Haque et al. 1996)and intensity and type of immune suppression (Mozzanica et al. 1997). The incidence of PTLD varies from 0.8% to 20% and is low for renal transplantation recipients and high for liver transplantation (LTx) recipients, which reflects the more intensive use of immunosuppressive drugs in the latter, possibly in combination with the different EBV load in the transplanted organ (Evens et al. 2010). PTLD is most likely caused by iatrogenical suppression of T-cell activity in transplantation recipients, which leads to inadequate immune surveillance against EBV-induced proliferation of infected B cells (Fields et al. 1990).Currently there is no definitive treatment regimen for PTLD prevention. The most common treatments are immunosuppression tapering and antiviral medications. Antiviral medications have been shown to inhibit the lytic cycle of EBV replication *in vitro* (Stevens et al. 1999) and a number of studies have been conducted to demonstrate the benefit of antiviral medications in post-SOT patients. In the renal transplant population, Porcu et al. (Brink et al. 1997b) also demonstrated that immunosuppression reduction and concomitant administration of acyclovir could induce a durable complete remission of PTLD. Other retrospective studies (Wagner et al. 1992) have indicated a benefit for antiviral therapy in decreasing PTLD incidence. Thus, the availability of a simple and reliable assay for timely detection of PTLD in transplant recipients is a pressing need. In a previous study, Riddler et al. found an association between high EBV DNA copy numbers and PTLD in SOT recipients (Stevens et al. 1999). Recent studies indicated, pediatric SOT recipients were mostly EBV DNA negative or showed very low EBV DNA levels (Bai et al. 1997b). In a study, using a semiquantitative PCR, it was shown that not all SOT patients with elevated levels of EBV DNA developed PTLD, but all PTLD patients had high EBV DNA levels (Nalesnik et al. 1988). Although there might be differences in the threshold values related to each method, the emerging pattern indicates that PTLD is associated with a high EBV DNA load in peripheral blood, as one would expect when considering the proposed pathogenesis of this

disease. This finding is clinically relevant, and it appears most important to differentiate between patients showing self-limiting reactivations and patients progressing toward an aggressive disease.

2.1.3. Correlative study on EBV load with cytokine profiles in pediatric post transplant patients.

Although much is known about the molecular mechanisms involved in EBV replication and latency, the specific mechanism(s) controlling virus reactivation in the immunocompromised host, and the relative role of lytic versus latent infection in the development of PTLD, are uncertain. Even in the presence of a strong humoral and cell-mediated immune response to the virus, EBV infection persists for the lifetime of the host (Basgoz and Preiksaitis 1995). Low-grade replication of EBV in B cells in the oropharynx can occur simultaneously with predominantly latent infection of B cells in peripheral blood and lymphoid tissue. EBV infection of B cells can also result in cellular activation and immortalization (Thorley-Lawson 1988). Reactivation of EBV and the proliferation of EBV-infected B cells are controlled by the immune response to the virus, particularly the human leukocyte antigen (HLA)–restricted EBV-specific cytotoxic T cell response (Hanto et al. 1985). When this response is inhibited by immunosuppressive therapy, the usual state of equilibrium between the virus and host immune response is disrupted in favor of the virus; latently infected cells can then either undergo proliferation or enter the lytic cycle.

De novo EBV infection can also be transmitted to the transplant recipient by donor organs or blood transfusions in this setting (Haque et al. 1996; Alfieri et al. 1996).

A better understanding of the biology of EBV infection and the pathophysiology of posttransplant EBV-driven lymphoproliferation would result in a more rational approach to strategies for the prevention and treatment of this disorder. Specific issues that need to be addressed include the mechanism of EBV-induced cell transformation and virus reactivation from latency, the response of EBV-transformed B cells to physiologic stimuli, and further clarification of EBV-specific epitopes recognized by the immune system in vivo. Identification of cytokines or other stimuli (including other viruses) that may influence the lymphoproliferative process would be useful. Additional knowledge regarding the epidemiology of EBV infection after transplantation, including the role of virus burden, organ versus blood transfusion

transmission, co infection by other viruses (e.g., CMV and HCV), and super infection with a second EBV strain, would also assist in the targeting of preventive strategies.

2.1.4. Genotypic detection of Epstein Barr virus from clinically suspected viral retinitis patients.

Viral retinitis is an important vision-threatening infectious disease of the retina which can occur in both immuno competent and immuno compromized or immuno deficient (acquired immunodeficiency syndrome – AIDS)individuals (Biswas et al. 1988). In immunocompetent patients, acute retinal necrosis (ARN) has been recognised as a vision-threatening inflammation caused primarily by herpes group of viruses (Freeman et al. 1986; Culbertson et al. 1986).Epstein Barr virus have been described in various ocular inflammatory diseases including multifocal choroiditis in healthy patients (Raymond et al. 1987; Tiedeman 1987). In immunocompromized, various opportunistic viral infections can occur; the most common being cytomegalovirus (CMV) infection and EBV. Other less common viruses causing retinal infections include herpes simplex virus (HSV) and Varicella zoster virus (VZV) (Tiedeman 1987).Although viral retinitis can usually be identified by the fundus picture and circumstantial evidence of immuno suppression (use of immunosuppressive drugs or a diagnosis of AIDS), the precise diagnosis and identification of the virus can be done best only with the help of rapid and sensitive virological studies .

The etiology of viral retinitis was earlier established by demonstrating the presence of antibodies raised against specific viral agent in the aqueous humour and serum samples. With the advent of PCR, technique it was possible to detect the specific infectious agent associated with retinitis as antibodies raised against Herpes group of viruses cross – react due to the antigenic similarity among gene products of herpes simplex virus types A and B, Epstein-Barr virus, and cytomegalovirus (Balachandran et al. 1987; Lang et al. 2001). Epstein –Barr virus is one of the important viral pathogens described in vision threatening ocular condition namely, viral retinitis among immuno-competent patient. In immuno-compromised patients EBV and CMV has been predominantly reported as the causative agent associated with. With the advent of PCR it was possible to accurately identify the infective agent associated with the clinical condition (Sugita et al. 2013).

2.1.5. Study of novel immune regulatory pathway associated with Epstein Barr Virus (EBV) and the recombinant EBV protein mediated inflammation in retinal pigment epithelium (ARPE-19).

To determine the host immune response to these infections, an *in-vitro* study on immune response to EBV infections especially ocular acute retinal necrosis which causes devastating effects such as blindness, was designed using Retinal pigment epithelial cells. The necrotic process is due to be driven by CD4+ cells, macrophages, polymorphonuclear cells, B cells, and the inflammatory cytokines TNF-α and IFN-γ (Khyatti et al. 1991).

In the present study we have used human retinal pigment epithelial cells (ARPE-19) as an in vitro model to study the possible immune response mediated by EBV P23 and NA proteins in acute retinal necrosis condition. Both p23 and NA proteins were capable of inducing both pro and anti-inflammatory cytokine production and TLR mediated immune response was evident in the transcript level. We further studied TLR associated cell signalling mechanisms in retinal epithelial cells using EBV strain A (B 95-8 culture filterate) along with recombinant proteins of both lytic and latent stage (P23 and NA). Both P23 and NA exposure induced nitric oxide production, apoptosis, and necrosis and cell death in the later stages of inflammation. The retinal pigment epithelium (RPE) plays a critical role in the development and maintenance of adjacent photoreceptors in the vertebrate retina. ARPE-19 is a spontaneously arising human RPE cell line with normal karyology which forms polarized epithelial monolayers on porous filter supports. The cell line is established by selective trypsinization of a primary RPE culture resulting in a uniform population of highly epithelial cells which exhibit a strong growth potential. ARPE-19 has structural and functional properties characteristic of RPE cells in vivo and is valuable for in vitro studies of retinal pigment epithelium physiology.

ARPE cells can also express TLRs in the absence of any pathogen. Toll receptors TLR2 and TLR5, but not TLR1 and TLR4, were expressed under oxidative stress in RPE (Mukherjee and Bazan 2005). High glucose concentration can up-regulate the expression of TLR2 mRNA in ARPE-19 cells and promote the translocation of PKC-α from cytoplasm to cytomembrane through the PKC-α pathway. Real time PCR analysis revealed gene expression for TLRs 1–7, 9, and 10 in RPE cells. TLRs

1 and 3 were the most highly expressed TLRs. Protein expression for TLRs 2, 3, and 4 was observed on RPE cells (Kumar et al. 2004). ARPE-19 cells retain many of the characteristics of RPE cells, including functional tight junctionsand the ability to phagocytose rod outer segments (Dunn et al., 1996).

2.2. Gaps in Existing Research:

The diagnosis of EBV is difficult in situations where clinical diagnosis is suggestive but confirmative test proof is lacking. Detection of EBV by conventional serological methods is simple and rapid but lacks adequate sensitivity, whereas PCR is comparatively more sensitive and specific. Molecular diagnostics is increasingly important for diagnosis and monitoring of patients affected by EBV-related diseases. These diseases represent a wide spectrum of clinical manifestations, from transient benign infection to aggressive malignancies. As virus-specific treatments continue to be investigated, it becomes even more important to recognize these EBV-associated diseases so that proper clinical management decisions can be made.

Serology remains the gold standard for diagnosis of primary EBV infection in immunocompetent patients. Infectious Mononucleosis diagnostics in particular typically involve a combination of serological markers as heterophile antibodies (HA), IgM antibodies against Viral Capsid Antigen (VCA IgM), VCA IgG and IgG against Nuclear Antigen (EBNA IgG). Nonetheless, despite the availability of several different serological markers, serology is not always able to accurately determine the stage of infection since false positive and false negative results are regularly observed. VCA IgM tests are useful in diagnosing patients with clinical suspicion of primary EBV infection but false negative results may occur due to the transient nature of the VCA-IgM response. Conversely, false positive IgM reactions occur due to autoantibodies or other serum factors and due to cross-reactions to other recent infections. There is thus a continuous quest for novel diagnostic methods that can enhance accuracy, including molecular assays as EBV Polymerase Chain Reaction (PCR). Thus, the present study was undertaken mainly to develop rapid, reliable and sensitive molecular techniques for the differential diagnosis of EBV directly from clinical specimens by nested PCR (nRT-PCR) and to study the prevalence of EBV circulating in Chennai population.

Serology is unreliable as a diagnostic tool for either Post transplant lymphoproliferative disorder (PTLD) or primary EBV infection in immunocompromised patients. The detection and quantification of EBV-DNA load in peripheral blood will serve as a prognostic marker for the development of PTLD, showing a correlation between high levels of EBV-DNA in the blood and the development of PTLD. Studies on detection of EBV and genotyping among pediatric population who have undergone SOT are limited and none from India. Therefore this study aims at determining the association of EBV in pediatric transplant patients and to know the most commonly present genotype among them.

Several researchers have developed Reverse Transcriptase based Real Time PCR, assays for detection and quantization of EBV RNA. Information is still sparse on EBV in PTLD, and the cytokine production in such condition needs to be further elucidated. This study provides rapid, reliable and sensitive molecular techniques for the detection of viable EBVfrom clinical specimens by Reverse Transcriptase real time PCR and its association with cytokine profile in post transplant pediatric patients.

Though there may reports available on application of PCR for detection of EBV and other herpes viruses from intraocular fluids of patients, no reports are available of the genotype associated with. Analyzing the genotype of EBV associated with viral retinitis will aid in understanding the phylogeny of the virus and also to know whether multiple genotypes are associated. In normal healthy individuals detection of EBV genotype A is reported and so in Infectious mononucleosis. In malignant conditions EBV B is reported. So far the genotypic prevalence of EBV among ocular clinical specimens collected from patients otherwise healthy and immnuo-compromised have not been studied. Therefore the current study was undertaken to determine the rate of detection of EBV and also to find the genotypic prevalence among them. To our knowledge, this is the first report investigating the presence of EBV DNA and Genotyping in intraocular fluids of patients with retinitis.

Epstein Barr Virus is associated with various ocular diseases like keratitis, uveitis and in some rare cases Acute Retinal necrosis Syndrome (ARNS) leading to retinal inflammation and detachment causing visual loss. The exact mechanism by which EBV causes retinal necrosis and the key viral proteins involved in the pathogenesis

are yet unknown. Therefore, in this study we have studied the possible pathogenic mechanism by which EBV can infect ARPE cells *in-vitro c*ausing inflammatory response and whether viral replication is involved in the development of inflammation in the eye as well as to gain a better understanding of immunological response of ARPE-19 treated with EBV strain-A (B95-8) and recombinant EBV proteins (Nuclear Antigen and p23).

2.3. OBJECTIVES:

2.3.1. Detection of Epstein - Barr virus in peripheral blood specimens obtained from patients with typical or suspected Infectious Mononucleosis known to be caused by EBV and to correlate the results with the serological and clinical findings.

- To perform ELISA for detection of antibodies raised against EA, VCA, EBNA of EBV on serum samples collected from patients with suspected infectious mononucleosis.
- To perform ELISA on serum samples collected from healthy voluntary blood donors and to estimate the seroprevalence among healthy population.
- To standardize nested PCR targeting VCA and EBNA1 gene for the detection of EBV.
- To apply the standardized PCR for detection of virus in direct specimens.

2.3.2. Detection of EBV on samples collected from pediatric post transplant patients and to study the genotype prevalence of EBV and to correlate the genetic characters (genotypes) with the clinical findings in the study population.

- To detect EBV on the samples collected using VCA and EBNA1 PCR.
- To estimate the viral load on the samples collected using standardized Real time PCR.
- To standardize PCR targeting EBNA2 and EBNA3c genes of EBV
- To apply the standardized PCR on the collected samples for detection of genotypes.
- To correlate the PCR results for detection of EBV genotype prevalence among pediatric post transplant patients.

2.3.3. Correlative study on EBV load with cytokine profiles in pediatric post transplant patients.

- To perform real time PCR for determination of viral load on blood samples collected from post transplant patients.
- To perform cytokine ELISA array for quantification of cytokines elevated during infection.
- To correlate the real time PCR results with cytokine ELISA array to find out the immunological response during active EBV infection.

2.3.4. Genotypic detection of Epstein Barr virus from clinically suspected viral retinitis patients.

- To detect EBV on the samples collected using standardized PCR
- To estimate the viral load using Real time PCR
- To perform PCR for detection of other infectious agents (HSV, CMV, VZV) on the collected samples.
- To perform genotyping PCR for detection of subtype prevalence in ocular infections.

2.3.5. **Study of novel immune regulatory pathway associated with Epstein Barr Virus (EBV) and the recombinant EBV protein mediated inflammation in Adult Retinal Pigment Epithelium (ARPE-19).**

- To determine whether EBV can infect ARPE cells *in-vitro c*ausing inflammatory response using Real time PCR.
- To detect change in the expression of cytokines in the infected cells.
- Application of Real time PCR for quantification of Toll like receptors expressed post treatment with viral antigens and EBV – 1 strain.
- To measure cell viability and observe cell lysis of infected cells.
- Analysis of the results obtained and to predict the immunological events occurring in the retinal cells post treatment with EBV and recombinant viral antigens.

CHAPTER 3

Detection of Epstein - Barr virus in peripheral blood specimens obtained from patients with typical or suspected Infectious Mononucleosis known to be caused by EBV and to correlate the results with the serological and clinical findings.

3.1 Introduction:

Infectious mononucleosis caused by Epstein-Barr virus is one of the common infections reported among children and adults followed by Human Cytomegalovirus (González Saldaña et al. 2012). 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(Ikuta et al. 2003). 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 to the virus gets infected.

Antiviral agents namely acyclovir or valacyclovir are recommended to treat the children with severe infection or those who develop complications (Balfour et al. 2007). The most commonly used diagnostic criterion is demonstration of atypical lymphocytes in peripheral blood smear (Hess 2004). However, presence of atypical lymphocytes alone is not sufficient for confirmation of mononucleosis. Further serological confirmation by demonstrating presence of IgG and IgM antibodies produced against different EBV antigens is essential(Macsween and Crawford 2003).

Although serological investigations are preferred for diagnosis of EBV infection where the result of EBV serology presents a high degree of variability, serology provides rational criteria for interpretation of the results (Huang et al. 2013). The detection of antibodies is less useful in immunocompromised patients because of their immune system dysfunctions, and the fact that the type of antibody and its maintenance may vary over time depending on the dynamics of the disease, thus leading to atypical profiles (Hess 2004).

Thus PCR based detection of EBV in direct samples becomes essential and to be evaluated against serological methods in diagnosis of IM caused by EBV. The comparative applicability of both the methodologies will aid in distinguishing acute and latent EBV infection. In the current study PCR targeting VCA and PCR

targeting EBNA1 was evaluated against serological methods. The comparative applicability of both the methodologies to distinguish acute and latent EBV infection was determined.

3.2. Hypothesis:

The nested PCR targeting VCA and EBNA1 genes associated with active and latent stage of the virus respectively will result in early and differential diagnosis of early primary EBV infection in samples collected from patients suspected with IM.

3.3. Materials and Methods:

3.3.1. Patients and clinical specimens:

Sixty peripheral blood samples were collected from immuno-competent healthy volunteer donors and served as control. Donors belonged to age group varying between 17 to 20 years. A total of one hundred and eighty peripheral blood samples were collected between August 2010 to July 2013 from one hundred and eighty immuno-competent patients clinically suspected with infectious mononucleosis at a tertiary child care centre, Chennai.

The age of the patients ranges from 2½ months to 14 yrs. All patients had at least one of the EBV related symptoms namely fever, rash, lymphadenopathy, pharygitis, hepatomegaly or splenomegaly. Children with EBV associated malignant disease such as malignant lymphoma or chronic active infections were excluded.

Samples were collected after getting the informed consent from the patient / patient’s dependent. The study was approved by the institute’s ethics sub-committee. Clinical details were recorded by the clinician in the proforma made specifically for the study.

3.3.2: Enzyme Immuno Assay (EIA):

Human IgG and IgM antibodies against EBV-VCA were tested by EIA following instructions of the manufactures (Demeditec Diagnostics, Germany: EN ISO 9001 Certified Company). All the samples were tested in duplicate. The patients who had detectable IgM antibodies to VCA and 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.

3.3.3. DNA extraction:

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

3.3.4. Polymerase chain reaction:

3.3.4.1. Semi Nested amplification of the *EBV-VCA* gene

The semi nested PCR for detection of EBV-VCA gene was standardized using the EBV Standard StrainCulture infiltrate of Marmoset cell line infected with EBV B958 (National Eye Institute, Bethesda, USA). In brief, the protocol followed was10 microliters of positive control DNA elute was subjected to amplification of EBV-VCA gene. Primers targeting VCA gene was designed indigenously using Primer premier biosoft international, 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 given in table 3.1. All primers and PCR reagents were procured from Bangloore genei.

3.3.4.2: 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 µls of extracted positive control DNA was added to the first round PCR reaction mixture. The reaction mix was incubated in the thermalcycler as follows: denaturing the DNA at 94°C for 5 minutes followed by amplification for 30 cycles, by 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 and 72°C. For the second round 5µl of the first round product was added to the 50µl of the PCR mix containing 100 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 anotherone serves as 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. DNA extraction, PCR cocktail preparation, amplification and analysis of results were carried out in physically separated rooms to prevent contamination. Visualization of PCR product was done by subjecting 10 µL of amplified reaction mixture to electrophoresis on a 2% agarose gel and documented on gel documentation system (Vilber Lourmat, France).

3.3.4 3.Specificity of PCR for the detection of EBV-VCA gene:

Specificity of the primers was determined against DNA extracted from*Herpes Simplex Virus 1* - ATCC VR 733, *Herpes simplex virus 2* ATCC 753167, *Cytomegalo virus* - ATCC 169 , *Varicella zoster virus* - ATCC Oca strain, , *Human DNA (extracted from whole blood), Eubacteria (Propionibacterium acne lab isolate), Fungus (Candida albicans)* ATCC 90028. All the standard strains are maintained in the laboratory.

3.3.4.4. Sensitivity of PCR for the detection of EBV-VCA gene:

DNA was extracted from 200 µl of EBV Standard StrainCulture infiltrate of Marmoset cell line infected with EBV B958 (National Eye Institute, Bethesda, USA) and the concentration was primarily quantified using nanovue (GE healthcare). 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. Initial DNA concentration used for the serial dilution of the positive control was 0.01ng. From each dilution, 10µl of DNA was taken for PCR reaction. The least dilution showing PCR positivity and its corresponding DNA concentration was calculated and considered as the sensitivity of the PCR.

3.3.4.5. Nested amplification of the *EBNA1* gene:

Nested PCR was optimized and performed for all the samples collected targetingEpstein Barr Nuclear antigen 1 *(EBNA 1)* as before(Tsai et al. 2002). The primers used for the nested PCR amplification are mentioned in table 3.1.

3.3.4.6. Nested amplification of the *MTR II gene* of CMV (Sowmya et al. 2006):

Nested PCR for detection of CMV was performed on all samples collected targeting *MTRII* gene of CMV. The primers used for CMV PCR are mentioned in table 3.1.

Genes	Primers	PRIMER SEQUENCE (5'-3')	Expected PCR product (Base pairs)
VCA	EBV F I	TTTGGCGTCTCAGGCTAT	Round 1 : 172
	EBV PP R	CGTGGTCGTGTTCCCTCA	
	EBV PP F	CGGTGTAACTACCCGCAATG	Round 2: 126
	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	
mtr II	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	

Table 3. 1: List of primers used for amplification of genes those codes for *VCA, EBNA1* of EBV and *mtr II* gene of CMV.

3.3.5. Statistical methods:

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

3.4. Results:

3.4.1. Serology:

A total of 180 specimens from patients suspected with infectious mononucleosis were tested for the detection of IgG and IgM antibodies against VCA antigen of EBV by ELISA. Out of 180 samples forty five (25%) were tested positive only for IgM antibody, seventy five (41.6%) other samples were tested positive for both IgG and IgM, thirty nine (21.6%) other samples were tested positive only for IgG and twenty one other samples were tested negative for both antibodies as depicted in Table 3.2 .
Among 180 samples four samples (2.2%) were tested positive for CMV IgG positive and fifteen (8.3%) were tested positive for both CMV - IgG and IgM positive.
Among 60 control sera from healthy population tested, IgM Antibody to VCA was detected in nine samples (15%) and IgG Antibody to VCA in 35 samples (58%).

S. No	Serology	EBV	CMV
1	IgM+IgG-	45(25%)	-
2	IgM+IgG+	75(41.6%)	15
3	IgM-IgG+	39(21.6%)	4
4	IgM-IgG-	21(28.8%)	-

Table 3. 2: EBV and CMVserological results of 180 clinical samples.

3.4.2. Polymerase chain reaction:

The primers used for VCA (semi nested) and EBNA1 nested PCRs were sensitive to detect 0.001ng DNA (Figure 3.1) and specific for EBV alone (Figure 3.2).

Figure 3.1: Agarose gel electrophoretogram showing the analytical sensitivity of *EBV-VCA* gene: Initial DNA concentration used for the serial dilution of the positive control was 0.01ng in 5µl of DNA. NC2 and NC1: Negative controls, 10^{-1} – 10^{-7}: 10 fold serial dilutions of EBV PC DNA, PC: Undiluted positive control DNA of EBV B95-8 strain.

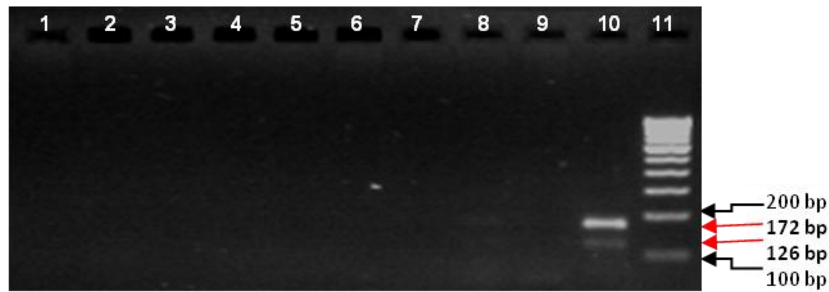


Figure 3.2: Agarose gel electrophoretogram showing the specificity of *EBV-VCA* gene:

1 and 2: Negative controls**, 3:** HSV1 DNAATCC VR 733**, 4:** HSV2 DNA ATCC 753167**,**

5: CMV DNA ATCC 169**, 6:** VZV DNAATCC OKa strain**, 7 :** Human DNA**,**

8: *Propionibacterium acne* Lab isolate**, 9 :***Candidaalbicans*DNAATCC 90028**,**

10 : Positive control DNA of EBV B95-8 strain**, 11 :** Molecular weight marker**.**

Among the 180 samples that were processed eighty nine (50%) samples were tested positive for *EBV-VCA* gene (Figure 3. 3) and eighty four (47%) were positive for *EBNA1* gene (Figure 3.4). Eighteen (30%) control samples collected from healthy donors were positive for EBNA1 PCR. None of the 44 healthy EBV – sero - positive blood donors (controls) had detectable gene encoding EBV VCA. Among 180 patients nineteen samples (10.5%) were tested positive for HCMV-*MTRII* gene.

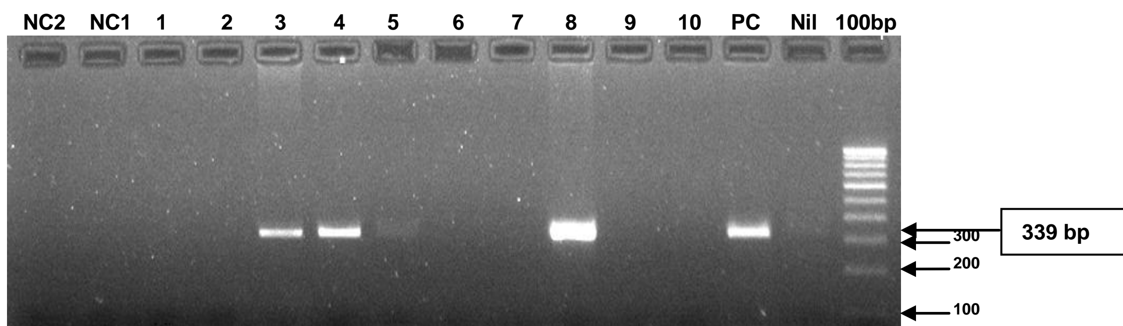


Figure 3.3: Agarose gel electrophoretogram showing EBNA1 PCR results of clinical samples:

NC2 and NC1: Negative controls, **Lanes 1-10:** DNA extracted from clinical samples, **Lane 3,4, 8:** EBNA1 PCR positive**, PC:** Positive control DNA, EBV B95-8 strain, **Marker 100bp:** 100 base ladder.

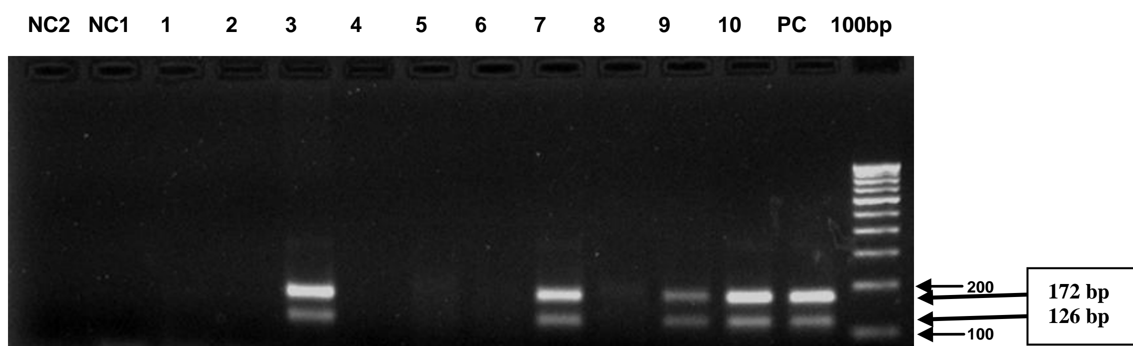


Figure 3.4: Agarose gel electrophoretogram showing EBV-VCA PCR results of clinical samples:

NC2 and NC1: Negative controls, **Lanes 1-10:** DNA extracted from clinical samples, **Lane 3, 7, 8, 9, 10:** VCA PCR positive, **PC:** Neat undiluted positive control DNA of EBV B95-8 strain, **100bp:** 100 base pair molecular weight ladder (100-1000)

3.4.3. Comparative study of EBV ELISA and PCR results:

Among the 180 samples processed 75 samples in whom both VCA IgG and IgM antibodies were detected 50 (66%) were tested positive for EBNA1 and 41 (54%) were tested positive for EBV-VCA PCR. Forty five other samples in whom only IgM antibodies were detected, 10 (22%) were positive for EBNA1 PCR and 41 (95%) others were positive for EBV-VCA PCR. Thirty nine other samples in whom only IgG antibodies were detected, 24 (61%) were tested positive for EBNA1 PCR and 7 (17%) were positive for EBV-VCA PCR. Samples that were sero negative (both IgG and IgM negative) were also detected negative by both VCA and EBNA1 PCRs. (Table 3.3).

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)	41/75 (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)

Table 3. 3: Correlation of EBV serological profiles with viral DNA detection by PCR**.**

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. P value for VCA PCR was calculated using SPSS software version 14.0 and was found to be <0.001, suggestive of highly significant.

3.4.4. Correlation of EBV and CMV ELISA and PCR results:

The false positive results of EBV-VCA IgM due to EBV/CMV antibodies cross reaction was confirmed by performing CMV ELISA and PCR. For better understanding and analysis of serological and PCR results the patients were categorized into four groups (Table 3. 4). Samples tested sero - positive for only IgM antibodies to VCA and tested negative by both EBV PCRs belongs to "Group A (n=4)". Samples tested sero-positive for IgM and IgG antibodies to VCA by ELISA and negative for VCA and EBNA1 PCR were categorized into "Group B" (n=34). "Group C" (n=15) consists of samples that were tested positive for IgG class of antibodies to VCA and negative for both PCR's. "Group D" (n=21) included samples that were both sero - negative and PCR negative for detection of EBV.

No. of samples	ELISA for EBV-VCA		ELISA for CMV		PCR Results		
	IgM	IgG	IgM	IgG	VCA	EBNA1	CMV
Group A, (No: 4)	+	-	+	-	-	-	+
Group B, (No:34)	+	+	+ **(n=18)**	-	+	+	+ **(n=18)**
Group C, (No: 15)	-	+	+	+	-	-	+
Group D, (No: 21)	-	-	-	-	-	-	-

Table 3.4: Correlation of EBV, CMV ELISA and PCR results on clinical samples.

Four samples belonging to group A was tested IgG positive for CMV ELISA and also found to have CMV DNA by PCR. Out of thirty four samples eighteen samples were found to be positive for IgM antibodies and PCR for CMV. Rests of sixteen samples under group B were negative by both IgM and IgG CMV ELISA and CMV PCR. Antibodies against CMV were tested positive in samples belonging to group C.

Fifteen other samples that were tested positive for IgG class of antibodies to VCA and negative for both PCR's were found to positive for both IgM and IgG CMV ELISA and also CMV PCR positive. Twenty one other samples categorized under group D was tested negative for CMV by both ELISA and PCR (Table 2.4). The HCMV PCR results were reproducible in all samples tested positive and negative.

3.4.5. Clinical signs and symptoms:

All the 180 patients included in the study were suspected with infectious monucleosis with presentation of high grade fever, and cough and all the 180 patients included had shown either of the criteria of Infectious Mononucleosis namely Lymphocyosis or presence of atypical lymphocyte in the blood smear study. Among the 180 patients, a total of 45 sero - positive (VCA IgM positive) patients presented with decreased oral intake, pharyngitis lymph adenopathy, hepato spleenomegaly, Abnormal Liver function test, Rhinorrhea etc in addition to fever and cough. The most common clinical symptom observed among patients with early primary infection were high grade fever and decreased oral intake (Figure 3.5).

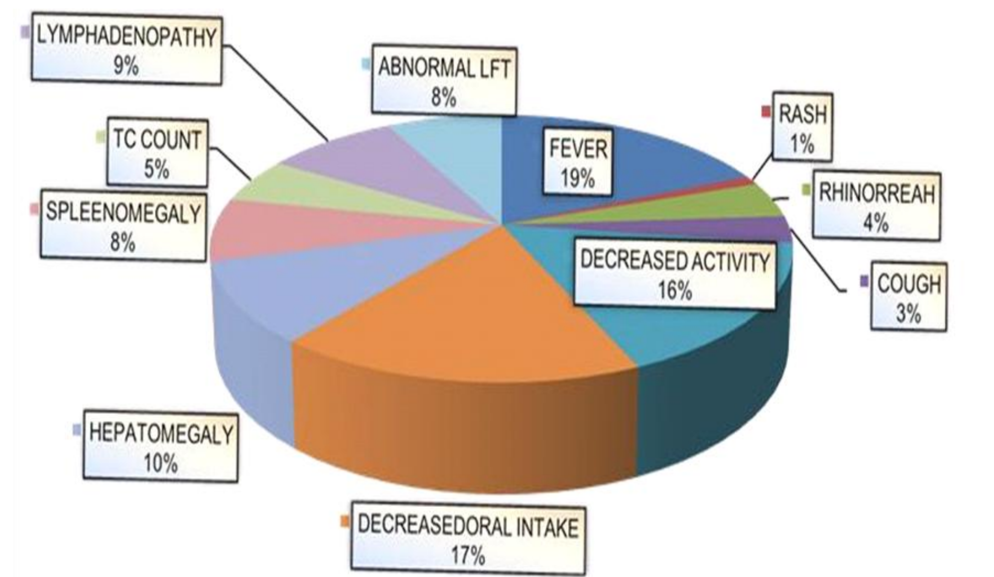


Figure 3.5: Clinical symptoms of patients included in the study.

3.5. Discussion:

Though serological tests are considered a reliable tool by clinicians in the diagnosis of infectious mononucleosis caused by EBV these do have demerits. IgM titre declines or disappears within 4 weeks. EBV infection can be mistaken for HCMV infection or streptococcal, throat infection or an ordinary fever, strictly because of the symptoms overlap.

Earlier studies have concluded neither a test of EBV VCA IgM nor a test of the presence of VCA IgG in the absence of EBNA 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 (Chan et al. 2001).

Using the standard serological profile as the reference Gold standard, in our study EBV-VCA gene was detectable in the 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 VCA PCR remain negative in sero - positive patients and sero- positive healthy donors in the absence of active EBV infection. The EBV/ CMV cross reaction as the reason for false positive EBV – VCA IgM reactions were confirmed by performing PCR targeting MTRII gene of CMV. Samples tested positive by EBV ELISA while negative by both EBNA1 and VCA PCR's were found to positive for CMV PCR and also both IgM and IgG antibodies were detected in these samples. Therefore in the current study the standardized VCA PCR helped to detect the EBV false IgM sero - positives.

The comparison of clinical data of patients diagnosed for early primary EBV infectionbased on PCR assay and serology revealed that high grade fever and cough were observed in all groups. Of the clinical signs presented among our study patientsdecreased oral intake, pharyngitis, lymph adenopathy, hepato spleenomegaly, abnormal, altered liver function and rhinorrhea were seen observed only among the 45 sero - positive (VCA IgM positive) patients (with early / primary infection)in addition to fever and cough observed in other patients. No significant correlation of clinical symptoms was observed in 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 as a reliable method to rule out the

cross reactivity and differential diagnosis of EBV induced Infectious Mononucleosis from Infectious mononucleosis – like syndrome.

3.6. Conclusions:

PCR targeting two different genes of EBV *VCA* and *EBNA1* was standardized and compared with the ELISA (VCA IgM, IgG) results to diagnose the early stage of infection caused by EBV. VCA PCR was found to be sensitive for the detection of EBV DNA in the early or primary stage of infection and can be considered as a reliable method to rule out the cross reactivity and differential diagnosis of EBV induced IM from IM– like syndrome.

CHAPTER 4

Detection of EBV on samples collected from pediatric post transplant patients and to study the genotype prevalence of EBV and to correlate the genetic characters (genotypes) with the clinical findings in the study population.

4 .1. Introduction:

Post-transplant lymphoproliferative disorder (PTLD) is a well recognized, although relatively uncommon, complication of both solid organ and allogeneic bone marrow transplantation (Gottschalk et al. 2005). The Epstein-Barr virus (EBV) is recognized as a primary pathogen causing infectious mononucleosis, a usually benign lymphoproliferative disorder most prevalent in adolescents and young adults. In most cases, PTLD is associated with EBV infection of B cells, either as a consequence of reactivation of the virus post transplantation or from primary post transplantation EBV infection acquired from the donor (Maeda et al. 2009). Like Human cytomegalovirus (HCMV), EBV maintains latency (by establishing persistent infection in B cells), which is controlled by virus-specific cytotoxic T lymphocytes (CTLs). The profound impairment of cytotoxic T lymphocyte response in solid organ transplant (SOT) recipients could result in failure to maintain latency, leading to uncontrolled EBV-driven B-cell proliferation and PTLD (Sebelin-Wulf et al. 2007). Though EBV has been associated with PTLD, a few detailed studies involving large number of pediatric patients has been carried out and none from India (Cui et al. 2011).

Serology is unreliable as a diagnostic tool for either PTLD or primary EBV infection in immunocompromised patients. PCR is an attractive diagnostic tool in this setting because of its ability to detect even fewer viral copy numbers.The detection and quantification of EBV-DNA load in peripheral blood have been utilized as prognostic markers for the development of PTLD, showing a correlation between high levels of EBV-DNA in the blood and the development of PTLD. Some patients show a marked delay in their humoral response to EBV antigens, and many fail to develop immunoglobulin (IgM) antibodies altogether (Tsuchiya 2002). Studies on detection of EBV and genotyping among pediatric population who have undergone SOT are limited. Therefore this study aims at determining the association of EBV in

pediatric transplant patients and to know the most commonly present genotype among them.

4.2. Hypothesis:

Significant proportion of children develops EBV infection following transplantation. PCR targeting two different genes EBNA3c and EBNA2 aids in detection of specific genotype and Type A EBV was found to be most prevalent. This study provides useful insights into the molecular epidemiology of EBV infection in India.

4.3. Materials and methods:

4.3.1. Patients and clinical specimens:

Seventy peripheral blood samples were obtained from 70 pediatric patients who had undergone transplantation. Of the 70 pediatric patients included in the study, 24 were renal transplant patients and 46 were liver transplant patients. The samples were collected after getting the informed consent from patient's kin. The study was approved by the institute's ethics sub-committee. Clinical details were recorded in the performa made specifically for the study. Two - three ml samples of peripheral blood was collected EDTA coated vacutainers and was processed immediately for separation of Buffy coat and was stored at -80°C for PCR until further processed. Sixty peripheral blood samples were collected from 60 healthy volunteer donors and served as control. The age of control group used ranged between 17 - 20 years. Lymphoid tissue biopsy sample was also collected from a patient with post transplant lymphoproliferative disorder. All the samples were subjected to viral load determination by real time PCR and genotyping by type specific PCR.

4.3.2. Positive controls:

4.3.2.1.EBV Standard Strain Type A: B958 cell line (NCCS, Pune)

4.3.2.2. EBV Standard Strain Type B: Culture infiltrate of Ag876 cell line (kind gift from Dr Alan Rickinson, Glasgow University, UK).

4.3.3. DNA extraction: DNA was extracted from standard strains, test and control blood samples and lymphoid tissue biopsy sample following the manufacturer's instructions of QIAGEN DNA extraction kit, Hilden, Germany. The tissue was

digested with ATL buffer (Tissue lysis buffer) (Qiagen) and proteinase K at 56°C for ten minutes. After proteinase K treatment, the DNA extraction was carried out with the manufacturer's instruction.

4.3.4. Nested PCR for detection of EBV:

In order to confirm the presence of EBV, two PCR's targeting the genes that codes for EBV-VCA and EBNA1 were applied on all 70 clinical samples. Uniplex PCR for detection of EBNA2, EBNA3C genes was standardized using the EBV-A and EBV-B Standard Strains. In brief, the protocol followed was 10 micro liters of DNA elute was subjected to amplification of the EBV specific genes mentioned. 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 4.1. All primers and PCR reagents were procured from VBC – Biotech service, Vienna.

Gene	Primer	PRIMER SEQUENCE (5'-3')	Expected Base pair
EBNA2	EBNA-2 F	TTTCACCAATACATGAACC	Type A:378
	EBNA-2R	TGGCAAAGTGCTGAGAGCAA	Type B:483
EBNA3C	EBNA3C-F	AGAAGGGGAGCGTGTGTTGT	Type A: 153
	EBNA3C-R	GGCTCGTTTTTGACGTCGGC	Type B: 246

Table 4.1: List of primers used for amplification of genes those codes for EBNA2 and EBNA3C.

4.3.5. Optimization of PCR:

All the PCR's were optimized to be carried out in the same thermal profile using gradient PCR. The PCR mixture contained 100 mM of dNTP mixture, 10X (5 µl) PCR buffer with 15mM $MgCl_2$, 1 µM of each forward and reverse primer and 3U/µl Taq DNA polymerase. Ten µls of extracted DNA was added to the PCR reaction mixture. The reaction mix was incubated in the thermalcycler as follows: denaturing the DNA at 94°C for 5 minutes followed by amplification for 30 cycles, by

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 and 72°C. 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/mlof ethidium bromide in 1X Tris-Borate buffer (pH -8.2-8.6) and documented on gel documentation system (Vilber Lourmat, France).

4.3.6. Specificity of PCR:

Specificity of the primers was determined against DNA extracted fromHerpes Simplex Virus 1, Herpes simplex virus 2, Cytomegalo virus, Varicella zoster virus, *Chlamydia trachomatis, Toxoplasma gondii*, Human DNA (extracted from whole blood), Eubacteria (*Propionibacterium acne*), Fungus (*Candida albicans*) .

4.3.7. Sensitivity of PCR:

DNA was extracted from EBV Standard Strain. Serial tenfold dilutions of the DNA starting from 10^{-1} to 10^{-10}, made from the extracted DNA by adding 5μl of DNA with 45μl of Milli Q water and transferring 5μl of the diluted DNA to the next series of vials. Initial DNA concentration used for the serial dilution of the positive control was 0.63ng. Ten μl were taken from each dilution for PCR reaction.

4.3.8. Real time PCR for Determination of viral load in clinical samples:

The viral load was estimated in the DNA extracts of all test and control samples using a commercial kit - "RoboGene Quantification Kit (Hilden, Germany) and 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 50°C for 30 minutes followed by initial denaturation at 95°C for 15 minutes and 50 cycles each consists of initial denaturation at 95°C for 30 seconds, annealing at 50°C for 60 seconds and extension at72°C for 30 seconds. The viral load was expressed in copies/ml of sample.

4.3.9. PCR for detection of genes that code for VCA, EBNA1:

The standardized PCR were applied on to the DNA extracts of 70 peripheral blood samples collected. The samples that are confirmed positive by both conventional and real-time PCR were further subjected to genotyping by PCR based DNA sequencing.

4.3.10. PCR for detection of genes that code for EBNA2 and EBNA3C of EBV:

Samples that are found positive for EBV were subjected to genotyping by PCR targeting EBNA2 and EBNA3C genes.

4.3.11. DNA sequencing:

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 PCR product. Amplification was carried out in 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 to confirm the sequenced data with standard strains, to determine the homology percentage.

4.3.12. Phylogenetic analysis*:*

The nucleotide sequences of the EBNA2 and EBNA3C PCR positive amplified products were analyzed by comparison with EBV standard strains nucleotide sequences. The nucleotide sequences were analyzed using BIOEDIT software. Evolutionary distances were estimated by constructing phylogram using UPGMA algorithm with perform bootstrap analysis (Replicates 1000) in CLC Main Workbench 6.71 software.

4.3.13. Statistical Methods:

The statistical significance of PCR on diagnosis of EBV in transplant patients was detected using Fisher's exact test. Mean, Standard deviation, median and Box plot for viral load and all statistical parameters were determined using SPSS14.

4.4. RESULTS:

4.4.1. Results of VCA and EBNA1 PCR:

Among the seventy clinical samples, thirty five (50%) were tested positive for EBV by both VCA and EBNA1 PCR. Thirty five other samples were negative by both PCR's. The sensitivity of both nPCR targeting VCA and EBNA1gene found to be statistically significant (Fisher's exact test < 0.05).

4.4.2. Analytical sensitivity and specificity primer set used for EBNA2 PCR:

EBNA2 PCR was sensitive to pickup 6.3fgamount of EBV DNA. Figure 4.1 shows the results of sensitivity and figure 4.2 shows the results of specificity of EBNA2 PCR.

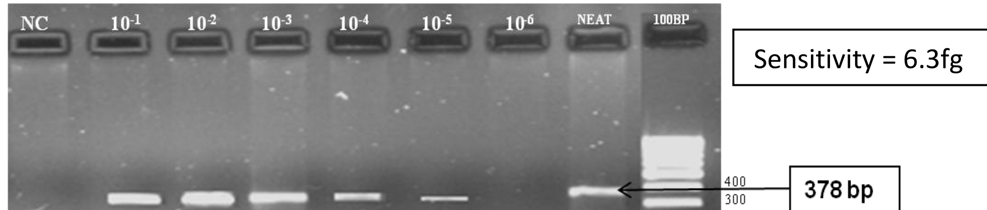


Figure 4.1: Agarose Gel Electrophoretogram Showing Sensitivity PCR for *EBNA2* gene of EBV.

NC: Negative control, $10^{-1} – 10^{-6}$: 10 fold serial dilutions of EBV PC DNA, Neat: Undiluted positive control DNA of EBV B95-8 strain, 100bp: Molecular weight marker (100bp – 1000bp). Initial DNA concentration used for the serial dilution of the positive control was 0.63ng in 5µl of DNA.

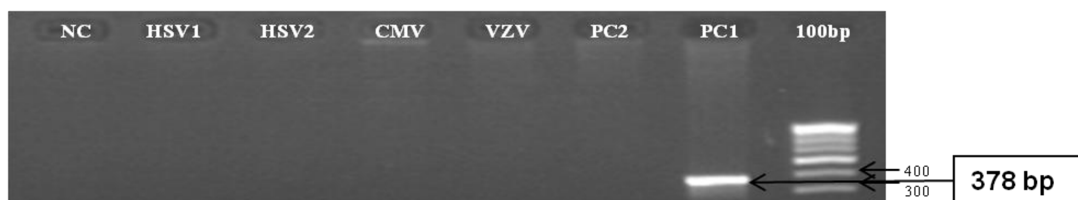


Figure 4.2: Agarose Gel Electrophoretogram Showing Specificity PCR for *EBNA2* gene of EBV.

NC: Negative control**, HSV1:** HSV1 DNAATCC VR 733**, HSV2:** HSV2 DNA ATCC 753167**, CMV:** CMV DNA ATCC 169**, VZV:** VZV DNAATCC OKa strain**, PC2:** Positive control DNA of

EBV Ag876 strain, **PC1:** Positive control DNA of EBV B95-8 strain**, 100bp:** Molecular weight marker**.**

4.4.3. Analytical sensitivity and specificity primer set used for EBNA3c PCR:

The EBNA3c PCR was sensitive to pickup 7.23fgamount of EBV DNA. Figure 4.3 shows the results of sensitivity and figure 4.4 shows the specificity of EBNA3c PCR.

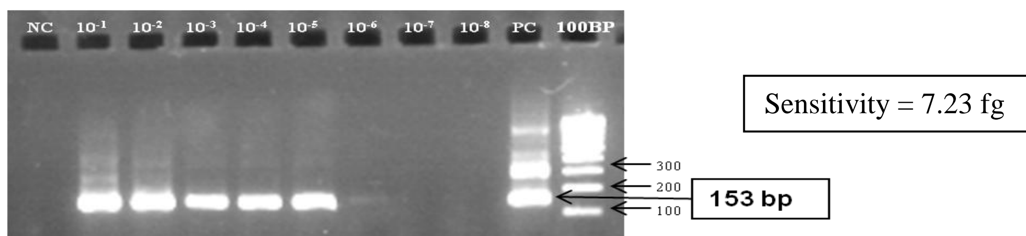


Figure 4.3: Agarose gel electrophoretogram showing sensitivity PCR for *EBNA3c* gene of Epstein - Barr virus. NC: Negative control, 10^{-1} – 10^{-8} : 10 fold serial dilutions of EBV PC DNA, PC: Undiluted positive control DNA of EBV B95-8 strain, 100bp: Molecular weight marker (100bp – 1000bp). Initial DNA concentration used for the serial dilution ($10^{-1}$to 10^{-8}) of the positive control was 0.723ng in 5µl of DNA.

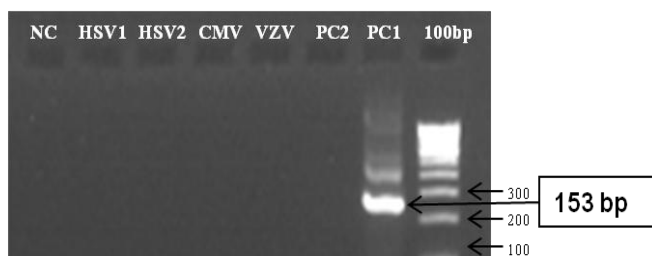


Figure 4.4: Agarose gel electrophoretogram showing specificity PCR for *EBNA3c* gene of Epstein - Barr Virus. NC: Negative control, HSV1: HSV1 DNAATCC VR 733, HSV2: HSV2 DNA ATCC 753167, CMV: CMV DNA ATCC 169, VZV: VZV DNAATCC OKa strain, PC2: Positive control DNA of EBV Ag876 strain, PC1: Positive control DNA of EBV B95-8 strain, 100bp: Molecular weight marker.

4.4.4. PCR-based Genotyping reveals the circulation of both EBV strains (Type A and type B) in Chennai population:

Genotyping was performed with DNA extracted from peripheral bloodsamples collected from transplant patients and control group that were tested positive by nPCR and real time PCR using PCR targeting EBNA2, EBNA3C genes. Type A was detected in thirty two (45.71%) and type B in blood of two (2.86%) samples. The

application of genotyping PCR to subtype EBV, revealed mixed subtypes (> one subtype) Co-infection with both A and B EBV genotypes was determined in one blood sample (1.43%) and also in the tissue sample (lymphoid biopsy) obtained from the same patient by both genotyping PCR. (Figure 4.5, Figure 4.6). The genotyping results in relation to patient's age and gender is listed in table 4.2. The genotyping PCR performed with the eight control samples that were tested positive for EBNA1 revealed EBV Type A.

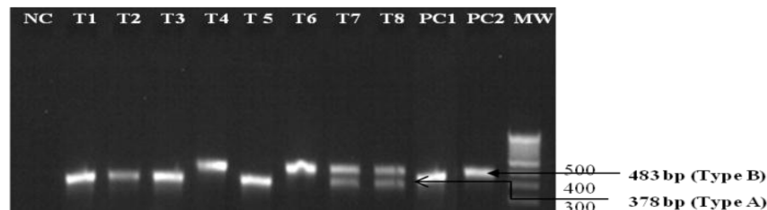


Figure 4.5: Agarose gel electrophoretogram showing amplification of EBNA2 gene of EBV in DNA extracted from blood samples of pediatric transplant patients. NC: Negative control, T1 – T8: DNA extracts from patient's samples, PC1: Positive control DNA of EBV B95-8 strain, PC2: Positive control DNA of EBV Ag876 strain, MW: 100bp ladder.

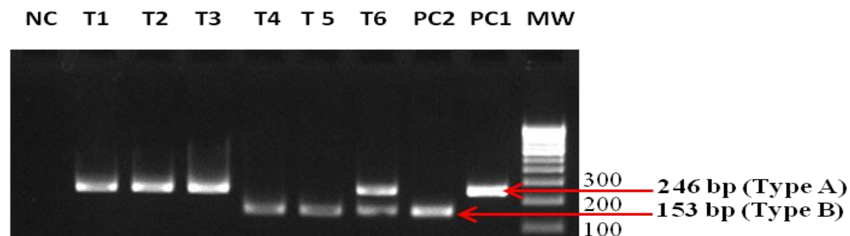


Figure 4.6: Agarose gel electrophoretogram showing amplification of EBNA3c gene of EBV in DNA extracted from blood samples of pediatric transplant patients. NC: Negative control, T1 – T6: DNA extracts from patient's samples, PC2: Positive control DNA of EBV Ag876 strain, PC1: Positive control DNA of EBV B95-8 strain, MW: 100bp ladder.

Genotype	Gender		Total of Samples
	Male	**Female**	
EBV –A	18 (51.43%)	14 (40%)	32 (91.43%)
EBV – B	2 (5.71%)	0	2 (5.71%)
Mixed infection	1 (2.86%)	0	1 (2.86%)
Total	21 (60%)	14 (40%)	35 (100%)

Table 4.2: Sample distribution in relation to patient's gender and EBV genotypes (n=35).

4.4.5. Determination of EBV viral load among patients samples:

Real time PCR was performed on all seventy patient's samples. All thetest samples detected positive by nPCR also tested positive by real-time PCR. The mean viral load for EBV positive patients who did not develop PTLD was found to be 50,424 C/ml (Lowest Viral Load: 14 C/ml and Highest Viral Load: 8, 20, 955 C/ml). The highest titer value of 89,14,188 C/ml was detected in the lymphoid tissue biopsy sample collected from a pediatric post liver transplant patient who was found to be co-infected by both genotypes of EBV. The real-time titre of EBV from the blood sample collected from the same patient was also significantly high (11,63,900 C/ml). This patient was found to have developed PTLD. Two samples positive for EBV Type-B genotype were found to higher titre values than all of the EBV type – A genotype positive sample (sample 3: 1,23,714 C/ml and sample 4: 8,20,955 C/ml). The real-time PCR values signifying the viral load in the samples that are positive are listed in Table 4.3. The statistical analysis (mean and standard deviation) performed on viral load quantification is provided in Table 4.4.Type A was detected in 32 patients and type B in 2 patients and co-infection with both A and B genotypes was determined in blood and tissue sample from one patient.

S No.	Age	Sex	Clinical Condition	Infection Type	Clinical specimen	Total WBC Count ~ 2 months Post Tx	EBV RT PCR (copies/ml)
1	1 Y	F	LT	A	Blood	10,400	478
2	1Y	M	LT	A	Blood	8,200	14
3	**10**	**M**	**LT**	B	**Blood**	7,100	**1,23,714**
4	**4Y**	**M**	**LT**	B	**Blood**	9,600	**8,20,955**
5	2Y	F	LT	A	Blood	9,200	607
6	3Y	M	LT	A	Blood	8,100	1,032
7	4Y	M	LT	A	Blood	5,300	1,40,392
8	14	M	RT	A	Blood	8,200	5,077
9	4Y	M	LT	A	Blood	4,600	216
10	1YF	F	LT	A	Blood	4,700	92
11	4Y	M	LT	A	Blood	10,500	1,559
12	4Y	M	LT	A	Blood	4,100	18,195
13	2Y	F	LT	A	Blood	4,300	768
14	2Y	F	LT	A	Blood	7,700	7,149
15	2Y	F	LT	A	Blood	13,300	15,715
16	4Y	F	LT	A	Blood	5,900	79,573
17	10M	M	LT	A	Blood	6,100	32
18	11M	M	LT	A	Blood	9,800	516
19	4Y	F	RT	A	Blood	7,400	98,464
20	3Y	F	LT	A	Blood	6,900	1,840
21	11Y	F	RT	A	Blood	7,100	840
22	4Y	M	LT	A	Blood	6,100	14,677
23	3Y	F	LT	A	Blood	4,500	1,505
24	11Y	F	LT	A	Blood	5,400	3,293
25	10Y	M	LT	A	Blood	8,100	62,124
26	1Y	F	LT	A	Blood	4,700	1,865
27	1Y	M	LT	A	Blood	8,000	58
28	2Y	M	LT	A	Blood	7,000	1,258
29	4Y	M	LT	A	Blood	8,000	1,20,179
30	12Y	M	RT	A	Blood	8,120	518
31	2Y	M	LT	A	Blood	7,390	465
32	2Y	F	LT	A	Blood	6,540	1,258
33	3Y	M	LT	A	Blood	9,200	1,78,420
34	4Y	M	LT	A	Blood	5,000	11,580
*35	4Y	M	LT	A + B	Blood	13,000	11,63,900
*36	4Y	M	LT	A + B	Biopsy	-	89,14,188

Table 4. 3: The clinical details of the patients who were tested positive by all PCR.

Note: M, Male; F: Female. LT- Liver Transplant, RT – Renal Transplant. * Sample 35 and 36 belong to same patient (Blood and Lymphoid tissue biopsy)

Genotype	No. of Samples	Mean Viral Load	Median Viral Load	Standard Deviation
EBV Type A	32	24054.97	1532	46969.53
EBV Type B	2	472334.5	472334.5	493023.84
EBV Type A and B	1	1163900	1163900	N/A

Table 4.4: Comparative analysis of viral load in EBV positive non PTLD patients

4.4.6. PCR results on control samples:

Eight (13.33%) among 60 control samples collected from healthy donors were positive for EBNA1 PCR. None of the 60 healthy donors (controls) had either detectable gene encoding EBV VCA, or the copy numbers quantified by real time PCR.

4.4.7. Sequence analysis on PCR amplified product of patients samples:

The results of genotyping PCR's were further confirmed by DNA sequencing using the PCR amplified products. The PCR amplified product of the samples (DNA extract of Blood and Tissue of same patient) showing the presence of both the genotypes A and B was subjected to gel elution using commercially available kit according to manufactures protocol (Qiaquick Gel elution kit, Hilden Germany) and individual gel eluted product were further confirmed by DNA sequencing.

4.4.8. Phylogenetic analysis of EBNA2 and EBNA 3C gene sequences confirmed circulation of both EBV genotypes:

The sequences were submitted to Genbank and the sequences were given the accession numbers from KC884748 – KC884757 and KF429681 – KF429706. 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 phylogram was constructed using DAMBE software. All the 32 samples that were confirmed as type A by PCR formed a unique clade with B95_8 strain. The two samples detected as type B by PCR formed a separate clade with EBV Type B standard strain Ag876 (Figure 3.7, 3.8). The results of phylogeny correlated with genotyping PCR results signifying the circulation of two different strains (EBV type A and EBV type B).

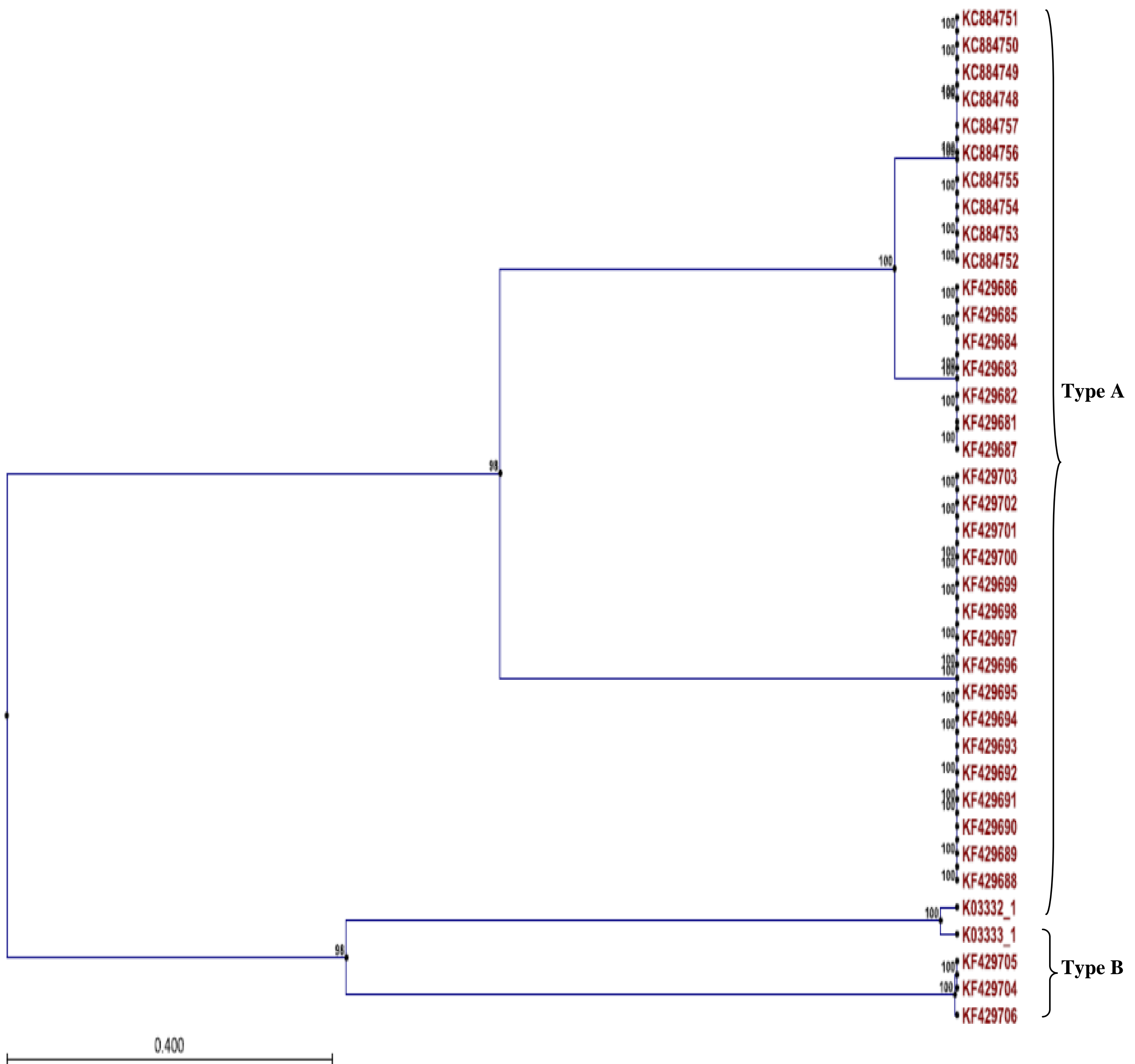


Figure 4.7: Phylogenetic analyses of the EBNA2 PCR positive samples from pediatric post transplant patients. The 483bp and 378bp sequences of EBNA2 gene of the representative samples were analyzed by the N-J method together with EBV type A (K03332_1) and EBV type B standard strain (KO3333_1) sequences. KC884748 – KC884757 and KF429681 – KF429706: Patient's test samples.

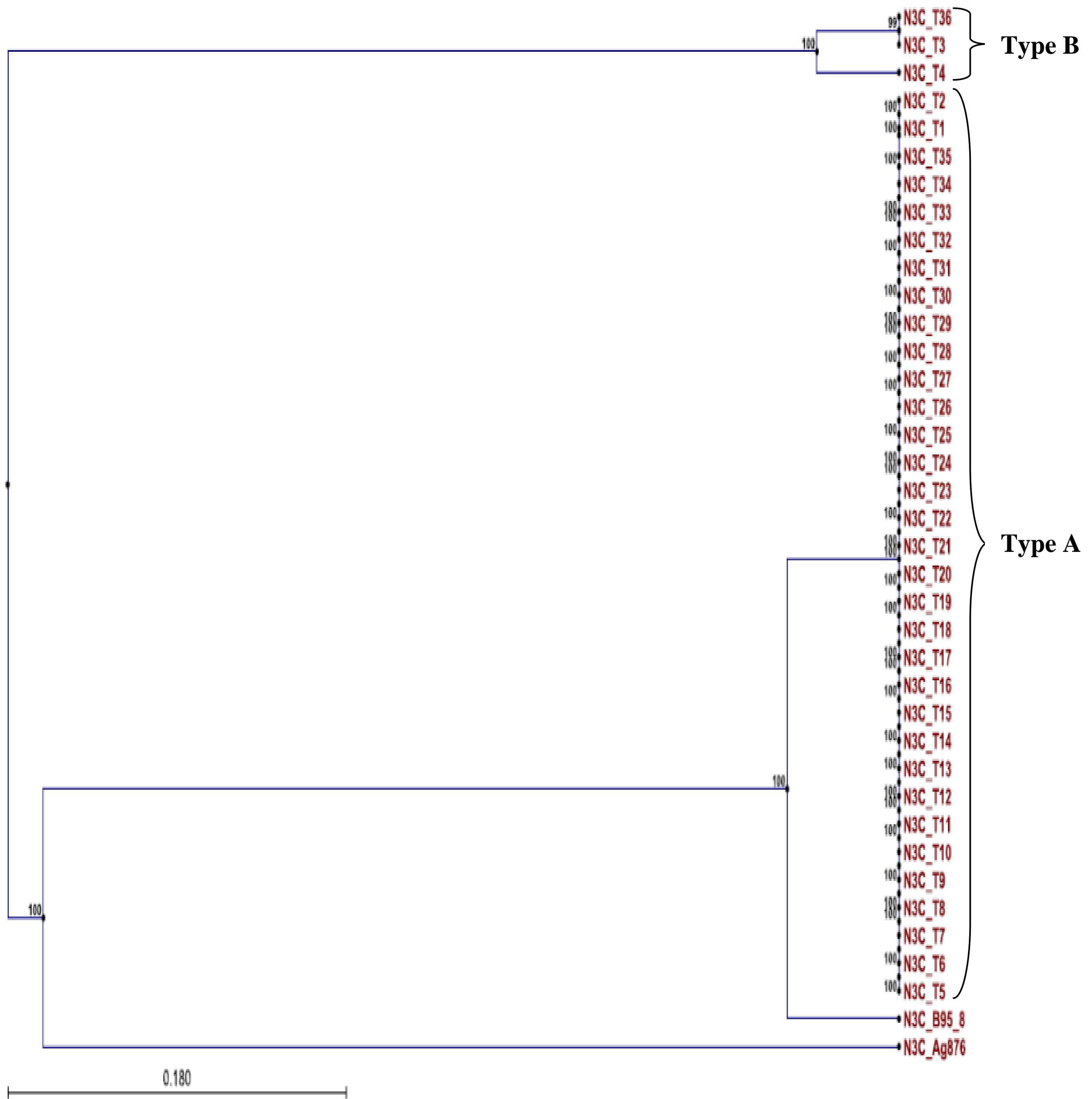


Figure 4.8: Phylogenetic analyses of the EBNA-3c positive samples from pediatric post transplant patients. The 153bp and 246bp sequences of a EBNA3C gene of the representative samples were analyzed by the N-J method together with EBV type A and EBV type B standard strain sequences.

4.5. DISCUSSION:

Though there many studies published on detection of EBV in PTLD, a few are available from pediatric population and none of these from India. The genotyping on EBV has never been attempted in pediatric post transplant patients. The identification of EBV strains has been used in many studies to investigate issues concerning the biology of infection and the nature of viral persistence (Sitki-Green et al. 2003).

Several studies have shown discrepancies between frequencies of EBV genotypes based on the analysis of certain genes such as EBNA-2, 3A, 3B, and 3C (Barzon et al. 2008). When applying PCR on viral DNA from cell culture isolation of clinical samples, in most of the cases, only one genotype, usually EBV-A, is detected. In contrast, when applying PCR directly on clinical samples co-infection with both EBV genotypes was observed. The discrepancies may be attributed to biological differences between genotypes, in which the EBV-A is more efficient than EBV-2 in the immortalization of growing B-cells in vitro. In attempt to avoid this tendency, the present study used PCR directly on the biological sample collected.

Types A and B can be determined by using the EBNA-2, -3A, -3B, or -3C gene. In the present study, we chose EBNA2 and EBNA-3C for genotyping. Previous studies on the classification of type A and B viruses have been based on the analysis of a single gene locus.

The present study has exploited the existence of two families of EBV strains that can be distinguished at two divergent gene loci (EBNA-2, EBNA-3).In the current study we have also standardized PCR targeting varying genes of EBV for genotyping and applied on clinical samples that are confirmed positive by both nPCR's and real time PCR. EBV was detected in 35 (50%) samples. Type A was detected in thirty two and type B in blood of two samples. Co infection with A and B EBV genotypes was determined in one blood sample and also in tissue sample from same patient. Though statistical analysis could not be applied on genotype prevalence, in our study, EBV type A was found to be more predominant in pediatric transplant patients as compared to EBV type B. Samples positive for EBV type B had significantly higher titre values than all of the EBV Type A samples. The sample with co-infection had the highest titre values and this patient also developed PTLD.

The highest prevalence of EBV-A occurs in the Western countries (Higa et al. 2002, Zhou et al. 2001). In this study, EBV-A was the most common genotype detected in 45.7% of the samples. In contrast to some studies, which proposed that in transplant patients there is high prevalence of the EBV type B infection or super infection with both type A and B (Okano et al. 1988), our study identified EBV-B and co-infection with both types in 2.86% and 1.43% of cases respectively. In this study, we monitored EBV viral load monthly in one liver transplant recipients for six months. The number of EBV-DNA copies was measured in peripheral blood sample by a quantitative PCR protocol. This patient developed PTLD and had recurrent acute illnesses during the study.

A significant proportion of children develop EBV infection following kidney or liver transplantation. Considering that 50% of the post-transplant patients we studied have been tested positive for EBV. Careful monitoring of EBV infection especially for cases with donor seropositivity is important to prevent disease progression. Early detection of PTLD is essential and it is best to have all post-transplant patients screened for EBV infections by PCR assays.

4.6. CONCLUSION:

PCR targeting varying genes of EBV and real time PCR can be applied to detect the genotype prevalence and to monitor development of disease and treatment. Type A EBV was the most prevalent subtype in pediatric cases post transplant and also there is a possibility of dual infection with EBV genotypes. This is the first time EBV genotyping based study is carried out in India.

Chapter 5

Correlative study on EBV load with cytokine profiles in pediatric post transplant patients

5.1. Introduction:

EBV infects over 95% of the world population (Cohen et al. 2000). Most infections occur in childhood and are asymptomatic; infection of adolescents and young adults with EBV often results in infectious mononucleosis. EBV is associated with a spectrum of lymphoproliferative diseases in patients with congenital or acquired immunodeficiency. Post-transplant lymphoproliferative disorders (PTLD) comprise a range of lymphoid tumors divisible into several clinicopathological categories (Okano et al. 2002, Straus et al. 1993). Most PTLDs are of B cell origin and contain the Epstein–Barr virus (EBV), which is considered to be a major cofactor for their development (Penn et al. 1969 and 1981).

EBV has evolved mechanisms utilizing its viral proteins to modulate host cytokine network in favor of its infection. First, it has been suggested that cytokines may serve to potentiate a host defense mechanism directed toward the eradication of virus. Second, cytokines play a cardinal role in the pathophysiology of EBV-associated infection (Ohga et al. 1999). The cytokine induction contributes to evade immune system and stimulates cell proliferation of specific immune cells. DNA identification of genes transcribed late during the infectious cycle of herpes viruses was used to indicate herpes virus active infection (Sabeti et al. 2003). EBV active infection leads to activation of mammalian cells and production of numerous cytokines and chemokines, which can induce further cell activation and cytokine production in a complex system of regulation and cross-regulation (Slots 2005; Stashenko et al. 1998).

5.2. Hypothesis:

Application of Nucleic acid based molecular techniques provides a rapid, sensitive, and reliable tool in the detection of EBV from clinical specimens. Presence of EBV by reverse transcriptase Real Time PCR will result in rapid detection of actively proliferatingEBV directly from clinical specimens.Using a multi-analyte ELISA array demonstrates the important role of cytokines expressed during acute EBV infection and suggests that these up regulated cytokines together with anti-EBV antibody

control EBV proliferation during acute EBV infection in Infectious Mononucleosis and post transplant patients and likely preventing PTLD.

5.3. Methodology:

5.3.1. Patients and clinical specimens:

The present study was approved by Ethics Committee of both the Research Institutes and informed consent was obtained from the patients who were enrolled in the study. The study subjects were categorized into three groups as Group I: Liver transplant patients, Group II: Patients with suspected Infectious mononucleosis, Group III: Other patients. Thirty peripheral blood specimens collected from liver transplant recipients (n = 10), Infectious mononucleosis patients, IMEBV (n=10) and others including healthy control individuals with no EBV infection NEBV (n = 10) were subjected to detection of antibodies to EBV, Real Time PCR and EBV induced innate immune response study consisting of estimation of Plasma levels of 11 anti-viral cytokines using multi analyte ELISA array.

5.3.2. RNA extraction:

RNA was extracted from the peripheral blood specimens using RNA extraction kit (QIAGEN, Germany) according to the manufacturer’s instructions. In brief, 280 µl of QIAGEN lysis buffer (AVL) was pipette into the bottom of a 1.5ml microfuge tube (DEPC treated) and 2.8 µl of carrier RNA was added. To this, 70 µl of the clinical specimen was added to the microfuge tube and mixed by pulse vortexing for 15 seconds. The specimen was incubated at room temperature for 15 minutes. 280 µl of ethanol (96-100%) was added to the sample and mixed by gentle pipetting for 15seconds. The mixture was carefully applied to the QIAamp mini spin column (in a 2ml collecting tube) and centrifuged at 8000 rpm for 1 minute. The spin column was placed in a clean 2 ml collecting tube and the tube containing the filtrate was discarded. 500 µl of buffer AW1 was added and centrifuged at 8000 rpm for 1 minuteand 500 µl of buffer AW2 was added centrifuged at 14,000 rpm for 3 minutes. For elution, 50 µl of buffer AVE was added and incubated at room temperature for 1 minute, followed by centrifugation at 8,000 rpm for 1minute the eluted RNA was used to determine the presence and determination of viral load by real time PCR.

5.3.3. Determination of viral load:

The viral load was estimated in the peripheral blood specimens using a commercial kit “RoboGene Quantification Kit (Hilden, Germany) as described in chapter 4 section 4.3.8.

5.3.4. Cytokine measurement in Serum samples:

The Multi-Analyte ELISArray analyzes a panel of 11 pro-inflammatory cytokines using a conventional ELISA protocol all at once under uniform conditions. The immune mediators detected were classified in 4 categories: (I) Proinflammatory mediators (Inter Leukine (IL)-A, IL-1B, IL-6, IL-8), (II) Type 1 cytokines (IL-2, IL-12, interferon [IFN]-γ, and tumor necrosis factor [TNF]–α), (III) Type 2 cytokines (IL-4, IL-10), (IV) T helper (Th) 17 cytokine (IL-17). Multi-Analyte ELISArray simultaneously profiles multiple cytokines / chemokines using simple ELISA protocol. The Multi-Analyte ELISArray method was performed as recommended by the manufacturer.

5.4 Results:

Antibodies to EBV were detected in 21 (70%) out of 30 peripheral blood samples. Out of thirty samples genomic EBV was detected in 15 samples (50%) by Real Time PCR. Among 30 samples included in the study antibodies to EBV were alone detected in 6 (20%) samples by ELISA but EBV RNA was not detected by RTPCR. The comparative analysis of detection of EBV RNA among different groups is provided in Table 5.1.

Groups	Genomic EBV Detected N=15	Genomic EBV not detected N = 15
Group I Liver transplant N= 10	7 (46.6%)	3[#] (20%)
Group II IM patients N = `10	8 (53.3%)	2[@] (15.3%)
Group III Control samples N = `10	0	10* (66.6%)

Table 5. 1: Comparative analysis of detection of EBV RNA among different groups

Note:

EBV antibodies were not detected by ELISA in all 3 serum sample obtained from a liver transplant patients. @ EBV antibodies alone were not detected by ELISA in 2 serum samples obtained from patients with infectious mononucleosis. * EBV antibodies alone were detected by ELISA in six samples obtained from Healthy control population.

The distribution of viral load in Group I: Liver transplant, Group II: Patients with suspected IM is provided in Figure 5.1 and 5.2 respectively. Mean and peak EBV levels were significantly higher in liver transplant recipients than patients with EBV induced IM like syndrome.

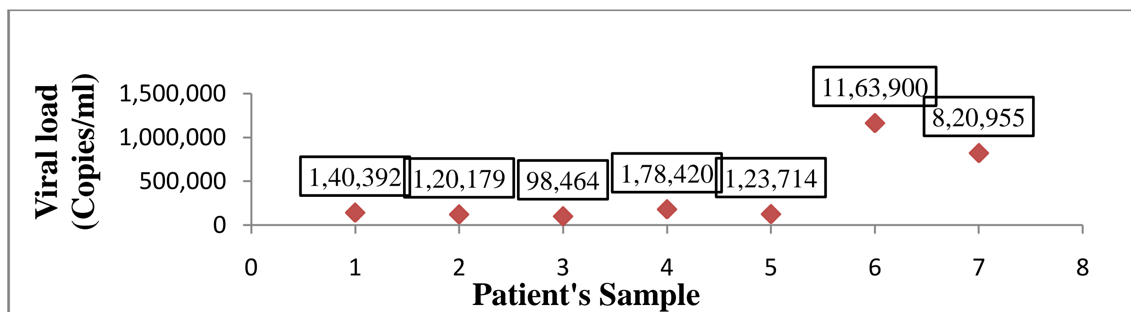


Figure 5.1: Scatter plot representing the viral load (C/ml) in Liver transplant patients (Group I: n = 7)

Scatter plot representing the viral load (C/ml) mentioned in the box for each patient's sample. EBV levels were significantly higher in liver transplant recipients than patients with EBV induced IM like syndrome. Patient (No. 6) who developed PTLD had very high copy number (11, 63,900) when compared with other patients.

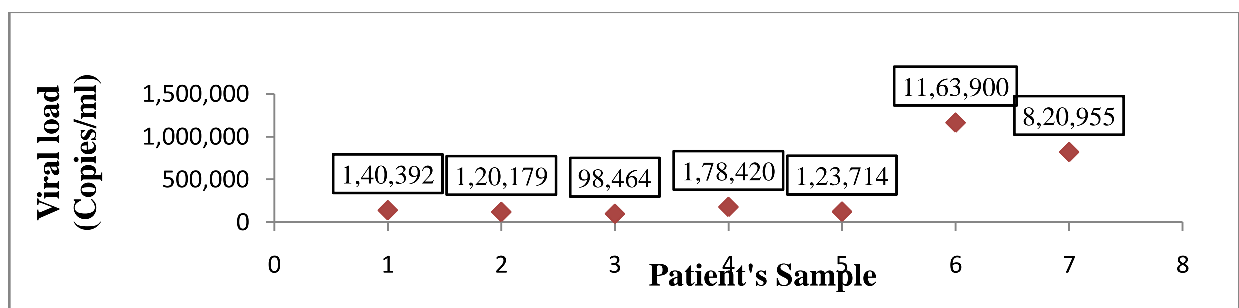


Figure 5.2: Scatter plot representing viral load in Infectious mononucleosis patients (Group II: n =8)

The statistical analysis performed on of cytokine parameters with respect to diseased and control groups on Cytokine expression in both EBV RNA positive and EBV RNA negative group using T test is provided in Table 5.2.

Category (A)	Category (B)	Mean Difference (A-B)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Group 1	Group 2	0.4373019	0.2248616	.144 (NS)	-0.117043	0.991647
	Group 3	1.9448455*	0.2248616	.000 (S)	1.390501	2.499190
Group 2	Group 1	-.4373019	0.2248616	0.144 (NS)	-0.991647	0.117043
	Group 3	1.5075436*	0.2248616	.000 (S)	0.953199	2.061889
Group 3	Group 1	-1.9448455*	0.2248616	.000(S)	-2.499190	-1.390501
	Group 2	1.5075436*	0.2248616	.000(S)	-2.061889	-0.953199

* *P**value < 0.05 statistically significant, NS: Not significant

Table 5.2: Comparative analysis of the mean and standard deviation of cytokine parameters with respect to diseased and control groups and Logistic regression analysis - Hosmer and Lemehow test performed on cytokine expression.

The IL-1b, IL-6 and IL-17A levels were significantly higher in post transplant patients than IM patients and NEBV. IFNg showed a similar trend, but not statistically significant. The IL-1b, IL-6 and IL-17A levels were significantly higher in EBV induced IM patients than NEBV but were significantly lower than those in transplant patients (Figure 5.3).

A logistic regression analysis was performed on cytokine elevation with EBV viral load. The inflammatory cytokines levels IL-6 and IL-17A were found to have a correlation with EBV viral load (**P* value < 0.05 statistically significant) and all the other cytokines were found to have no correlation or statistical significance. Among 7 post transplant patients one patient with persistent EBV developed PTLD with high levels of IL-2, IL-1B, IL- 6, IL-17A, IL-10 and TNFA in serum.

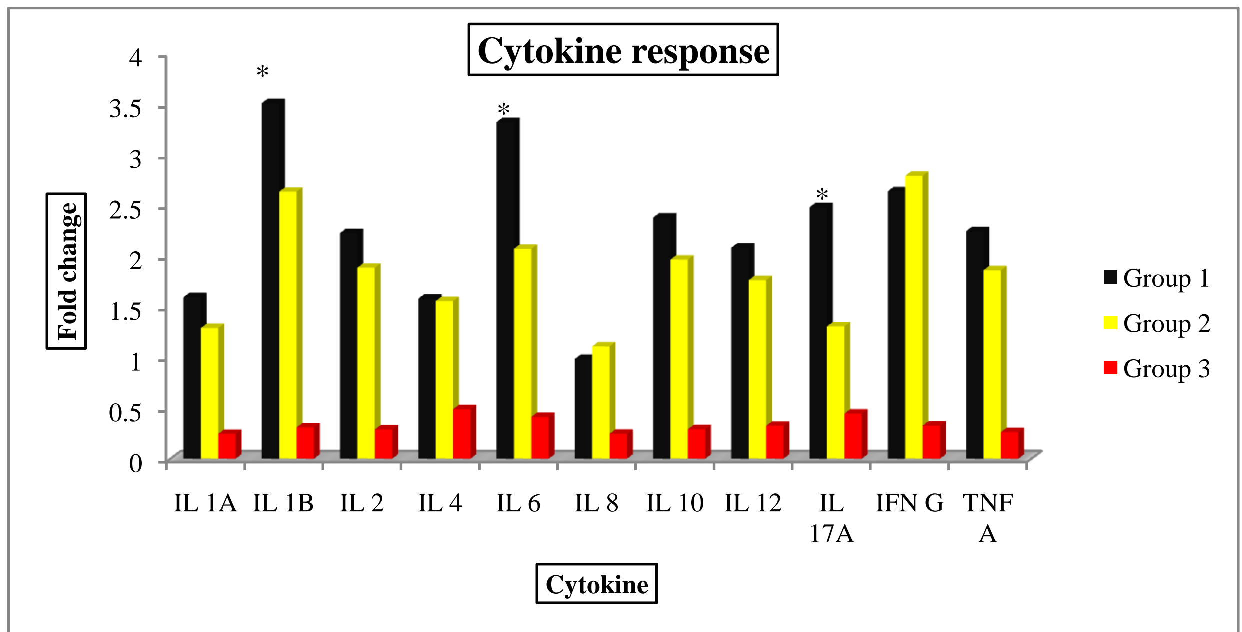


Figure 5.3: Quantification of cytokines in serum samples collected from post transplant, infectious mononucleosis and healthy control population. * *P**value < 0.05 statistically significant.

The levels of IL-1b, IL-6 and IL-17A levels significantly higher in post transplant patients (group 1) than Infectious Mononucleosis patients (group 2) and NEBV (non EBV/ healthy controls, group 3).

5.5. Discussion:

The present study provides the evidence that EBV participate in the pathogenesis of PTLD. Assuming that the amount of virus and cytokine production is an important parameter when evaluating a role in pathogenesis, quantitative real-time PCRs were performed in the present study. The difference in occurrence of EBV and cytokines between patients and control group was statistically significant. EBV RNA was not detected in the control group, which is in agreement with previous studies reported by Sabeti et al. 2003.

Semiquantitative PCR analysis of EBV DNA with use of mononuclear cells is useful for the diagnosis of EBV-associated diseases (Gustafsson et al. 2000). Kimura et al. have quantified EBV DNA in mononuclear cells of patients with symptomatic EBV infections by real-time PCR and have indicated the usefulness of the method for monitoring virus load (Kimura et al. 1999). EBV genome can be detected in cell-free samples of patients with posttransplantation lymphoproliferative disease or human immunodeficiency virus infection (Limaye et al. 1999; Portolani et al. 2009). The amount of viral DNA seems to

be a prognostic marker for chronic active EBV infection(Ohga et al. 2001; Kanegane et al. 1999).

The presence of viral RNA implies that active transcription occurs. This study clearly demonstrates the significance of active EBV infection (RNA expression) in key cytokine production. In this context, the comparative analysis in the result section exhibited a parallel marked increase in the cytokines and EBV expression in post transplant patients when compared with control population.

The elevation of IL-2 and IFN-g levels in serum samples of patients indicates the characteristic T-lymphocyte activation during active EBV infection in post transplant patients and patients with EBV induced infectious mononucleosis when compared to control population. IFN-g serves as an antiviral cytokine by inhibiting viral replication or eliminating viruses from infected cells.(Novelli and Casanova 2004)

In addition to the T-cell activation, we observed significantly larger amounts of TNF-a, IL-1b, and IL-6 in the serum sample of patients with high viral count when compared with healthy controls. We interpret these findings as signs of increased activity of monocytes during primary EBV infection, although other leukocytes could, at least partly, be responsible for the secretion of these cytokines; e.g., IL-6 is also produced by activated TH2 cells.

General cytokine effects of IL-6 and IL-1b are the induction of B-cell proliferation and differentiation. Furthermore, IL-6 enhances the differentiation of cytotoxic CD81 T lymphocytes in the presence of IL-2 or IFN-g (Bende et al. 1992) and regulates Ig gene transcription in B lymphocytes (Tanner and Tosato 1992) . Both effects could be demonstrated during the immune response to EBV. Moreover, TNF-a, IL-1b, and IL-6 induce hypothalamic prostaglandin E2 synthesis and fever, and IL-1 and TNF-a initiate the release of acute-phase proteins. Therefore, these cytokines may be responsible for clinical symptoms during EBV infection. Gene transcription of all three cytokines was shown to be strongly enhanced in lymphoid tissue during acute EBV infection in transplant patients and infectious mononucleosis (Foss et al. 1994).

Collectively, our results confirm monocyte-derived factors such as TNF-a, IL-1b, and IL-6 may play an important role in the pathogenesis of primary EBV infection. Increased levels of IL-1b and IL-6 may further explain B-lymphocyte proliferation and differentiation, and the synthesis of unspecific antibodies. Selective regulation of cytokine synthesis by EBV may, on the other hand, favour different pathological proceedings, such

as reactivation of other infectious agents. Knowledge about the pathogenic factors of PTLD and IM may help in the development prognostic markers and therapeutic strategies for treating chronic infectious mononucleosis and EBV induced PTLD in immunocompromised post transplant patients.

5.6. Conclusion:

EBV RNA levels were significantly higher during post transplant patients than IM and the presence of viral RNA implies that active transcription occurs. EBV viral load was not detected in control population. The levels of IL-1b, IL-6 and IL-17a were significantly high in post transplant patients than in IM patients and control population. Levels of IL-6 and IL-17a were significantly higher at high EBV DNA levels observed during PTLD and acute IM. This study clearly demonstrates the significance of active EBV infection (RNA expression) in key cytokine production.These results demonstrate an important role of IL-6 and IL-17a during acute EBV infection and suggest that these cytokines together with anti-EBV antibody control EBV proliferation during acute EBV infection in IM and post transplant patients and likely preventing PTLD.In addition to that elevated expression of anti-inflammatory cytokine (IL-10) was observed in the EBV^{+} (positive) samples in comparison with EBV^{-} samples (undetectable or negligibly low levels of expression) which indicate its relevance with viral persistence in EBV transformed tumour cells. These studies will open novel avenues to study cellular and/or viral gene expression and will introduce new biomarkers and strategies to improve clinical as well as diagnostic practices in chronic EBV infection.

Chapter 6

Genotypic detection of Epstein Barr virus from clinically suspected viral retinitis patients.

6.1. Introduction:

Herpetic ocular infection is a major cause of blindness worldwide. It is well known that herpes viruses including herpes simplex virus (HSV), varicella zoster virus (VZV) Epstein Barr virus (EBV) and cytomegalovirus (CMV) are involved in the pathogenesis of many ocular diseases including keratitis, keratoconjunctivitis, uveitis, iridocyclitis, acute retinal necrosis (ARN) syndrome and chorioretinitis (Yamamoto et al. 1996). When occurring in immunocompetent patients, these infections can often lead to diagnostic dilemmas due to a low index of suspicion. Further, there could be significant overlapping of clinical features, especially in the early stages of the disease leading to misdiagnosis. The initial diagnosis of these infections is very important under such circumstances since it determines the choice of antiviral drugs (Koizumi et al. 1999). Studies of Epstein-Barr virus (EBV) infections are limited by the lack of routine culture techniques. Currently, laboratory diagnosis of primary or reactivated EBV infection depends on serologic tests (Fleisher et al., 1983). However, serology is an indirect marker of infection and is difficult to interpret in the presence of immunosuppression (Katz and Saini, 1992; Okano et al., 1988). Establishing the presence of EBV would have a definite impact on our understanding of the spectrum of EBV disease, pathogenesis, and management of the increasingly common EBV related ocular infections (Schlossberg 1989). In addition, it could disclose associations of EBV to febrile illnesses and other poorly defined complications of immunosuppression (Telenti et al. 1990).

Polymerase chain reaction (PCR) is now commonly used for diagnosing viral diseases. Though PCR detects only the presence of viral DNA, it can often be correlated with current clinical conditions for effective therapeutic management. Further, PCR overcomes the lower sensitivity of conventional laboratory techniques such as antibody detection methods while maintaining the specificity. During the last decade, several studies have concluded that PCR based laboratory investigations are a valuable approach for achieving reliable diagnosis of viral ocular infections (Cunningham et al., 1996).

6.2. Hypothesis:

Polymerase chain reaction targeting VCA and EBNA1 genes are useful in detection of Epstein Barr virus from ocular samples. The application of genotyping PCR to detect genotypes can be used as an epidemiological tool for clinical management.

6.3. Materials and methods:

6.3.1. Patients and clinical specimens:

All patients and control population were recruited from Sankara Nethralaya, Chennai, India. The study protocol was approved by the Ethics Committee of the institute and the informed consent was obtained from each patients and control subjects. A total of seventy Aqueous Humor (AH) samples (20 AH from 20 HIV positive patients with clinical picture (necrosis, blurred vision, AC flare) suggestive of retinitis (Blind spot, blured vision and other vision problems, floaters) and atypical necrotizing retinitis and 25 AH from 25 patients with serpignous choroidoitis and 25AH from patients undergoing cataract surgery, which was considered to be the negative control) were collected from January 2012 to January 2013. The samples were processesed immediately and DNA was extracted and subjected for molecular assays.

6.3.2. Processing of Aqueous Humor (AH):

AH samples (150-200 µL) were collected aseptically in a tuberculin syringe with a 30-gauge needle, under aseptic precautions by the ophthalmologist; the sample was transferred onto a pre-sterilized microfuge tubes and stored at -20°C for DNA extraction.

6.3.3. Positive controls:

EBV Standard Strain Type A: Culture infiltrate of Marmoset cell line infected with EBV B958 (National Eye Institute, NIH, Bethesda, USA). **EBV Standard Strain Type B:** Culture infiltrate of Ag876 cell line (kind gift from Dr. Alan Rickinson, Glasgow University, UK).

6.3.4. DNA extraction:

DNA was extracted from standard strains, test and control samples following the manufacturer's instructions of QIAGEN DNA extraction kit, Hilden, Germany.

6.3.5. Polymerase chain reaction for detection of EBV:

In order to confirm the presence of EBV, two PCR's targeting EBV-VCA and EBNA1 were applied as previously mentioned (Table 4.1). The reaction mix and thermal profile previously mentioned (Chapter 4, Section: 4.3.5) was followed.

6.3.6 Real time PCR for Determination of viral load in clinical samples:

The viral load was estimated in the DNA extracts from AH samples using the RoboGene Quantification Kit (Hilden, Germany). The amplification reaction was carried out following the manufacturer's instructions. PCR was carried out at 50°C for 30 min followed by initial denaturation at 95°C for 15 min followed by 50 cycles of initial denaturation at 95°C for 30 Sec, annealing at 50°C for 60 Sec, and extension at 72°C for 30 Sec. The viral load was expressed in copies/ml. Samples that were positive for CMV by nested PCR were also subjected to CMV real-time PCR using the "Artus CMV RG PCR kit" on a Rotor-Gene 3000 instrument as per manufacture's instruction.

6.3.7.Genotyping PCR for detection of EBNA2 and EBNA3C genes of EBV:

The samples that are confirmed positive by both conventional and real-time PCR were further subjected to genotyping by PCR based DNA sequencing targeting EBNA2 and EBNA3C genes as previously mentioned in chapter 4 (Table 4.2).

6.3.8. Phylogenetic analysis:

The nucleotide sequences of the EBNA2 and EBNA3C PCR positive amplified products were analyzed by comparison with EBV standard strains. The nucleotide sequences were analyzed using BIOEDIT software. Evolutionary distances were estimated by constructing phylogram using UPGMA algorithm with perform bootstrap analysis (Replicates 1000) in CLC Main Workbench6.71 software.

6.4. Results:

Among the forty five tests clinical samples seventeen clinical samples (37.7%) were tested positive for EBV by both VCA and EBNA1 PCR. The samples that were detected positive for nPCR were also found to be positive by real-time. The Real-time PCR results were 100% correlating with the conventional PCR. The mean EBV viral load is 84310.35 copies/ml. The real-time PCR values signifying the viral load in the samples that are positive are listed in table 6.1.

S No.	Clinical condition	Quantiferon & CD 4 count	VCA PCR	EBNA PCR	EBV RT PCR	CMV PCR	CMV RT PCR	HSV1, HSV 2, VZV, Mtb & TOXO PCR
1	GHPC	P	**P**	**P**	**14**	N	N	N
2	HAC	N	**P**	**P**	**427**	N	N	N
3	SC	N	**P**	**P**	**607**	N	N	N
4	SC	N	**P**	**P**	**1693**	N	N	N
5	AC	N	**P**	**P**	**1032**	N	N	N
6	GHPC	N	**P**	**P**	**123714**	N	N	N
7	AMC	N	**P**	**P**	**820955**	N	N	N
8	GHPC	N	**P**	**P**	**140392**	N	N	N
9	SC	N	**P**	**P**	**5077**	N	N	N
10	GHPC	N	**P**	**P**	**249000**	N	N	N
11	SC	P	**P**	**P**	**14138**	N	N	N
12	SC	N	**P**	**P**	**1123**	N	N	N
*13	GHPC	N	**P**	**P**	**98**	N	N	N
*14	NR	120	N	N	N	**P**	**13295**	N
*15	NR	142	N	N	N	**P**	**102**	N
*16	NR	84	**P**	**P**	**16408**	**P**	**7504**	N
*17	NR	22	**P**	**P**	**42216**	N	N	N
*18	ANR	100	**P**	**P**	**2141**	**P**	**76**	N
*19	NR	76	**P**	**P**	**14241**	**P**	**146**	N

Table 6.1: The relevant clinical details of the patients and results of PCR tests performed with the aqueous humor for the infectious agents listed.

Note. GHPC: Giant Helicoid Peripapillary Choroidopathy, HAC: Healed ampiginous Choroiditis, SC: Serpiginous Choroiditis, AC: Ampiginous Choroiditis, AMC: Active Multifocal Choroiditis H1, Herpes simplex virus1; H2, Herpes simplex virus2; CMV, cytomegalo virus; VZV, varicella zoster virus; M. tb, Mycobacterium tuberculosis; T. gondii, Toxoplasma gondii; VCA Epstein Barr viral capsid antigen; EBNA1,

Epstein Barr nuclear antigen;.P, positive; N, negative. * HIV positive patients; NR, Necrotizing retinitis; ANR; Atypical necrotizing Retinitis.

Genotyping PCR targeting EBNA2 and EBNA3C genes on all clinical samples collected revealed the presence of EBV Type A in all seventeen samples that were proved positive by both nPCR and by real time PCR. EBV Type B was not found in none of the sample processed. The application of genotyping PCR to subtype EBV, revealed the circulation of only one subtype (Type A) in ocular sample. The genotyping PCR electrophoretograms of EBNA2 and EBNA3c on clinical samples are shown in Figure 6.1 and 6.2 respectively.

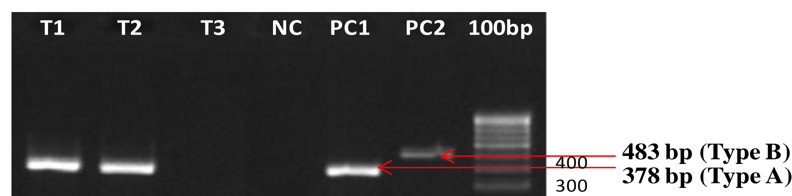


Figure 6.1: Agarose gel electrophoretogram showing amplification of EBNA2 gene of EBV in DNA extracted from AH sample.NC: Negative control, T1, T2: EBV Type A positive, T3: EBV PCR Negative, NC: Negative control, PC1: Positive control, DNA extracted from Type A standard strain - B95-8, PC2: Positive control, DNA extracted from Type B standard strain - Ag876, 100bp: Molecular weight marker (100-1000bp ladder).

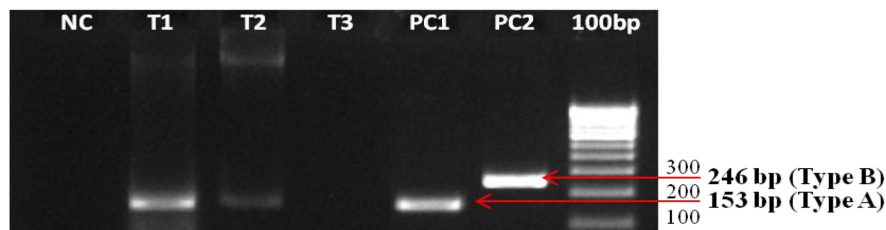


Figure 6.2: Agarose gel electrophoretogram showing amplification of EBNA3c gene of EBV in DNA extracted from AH sample. NC: Negative control, T1 – T3: DNA extracts from samples, T1, T2: EBV Type A positive, T3: EBV PCR Negative, PC1: DNA extracted from Type A standard strain - B95-8 PC2: Positive control, DNA extracted from Type B standard strain - Ag876, 100bp: Molecular weight marker (100-1000bp ladder).

The specificity of VCA was confirmed on 25 AH samples collected from patients who underwent cataract surgery because none of them were positive for these genes. PCR's were performed for other possible causative infectious agents of retinitis, *Mycobacterium tuberculosis*., *Toxoplasma gondii*, Herpes simplex virus 1, Herpes

simplex virus 2, cytomegalovirus, varicella zoster virus as shown in table 6.1. PCR results for the other infective agents were negative in all patients except for the presence of CMV DNA in 5 (20%) AH collected from HIV positive patients with clinically suspected viral retinitis.

The results of genotyping PCR's were further confirmed by DNA sequencing using the PCR amplified products. The sequences obtained were submitted to Genbank. The accession numbers are KF651147 - KF651163. The nucleotide sequences of the EBNA2 positive amplified products were analyzed by comparison with EBV standard strains nucleotide sequences. The sequences were aligned using BIOEDIT software and a phylogram was constructed using UPGMA algorithm with perform bootstrap analysis (Replicates 100) in CLC Main Workbench 6.71 software. All the 17 samples that were confirmed type A by both genotyping PCR found to form a unique clade with EBV Type A standard strain. The results of phylogeny correlated with genotyping PCR results (Figure 5.3). The fundus photography of patients with the highest EBV load, showing the typical chorioretinal lesions is given in figure 6.4.

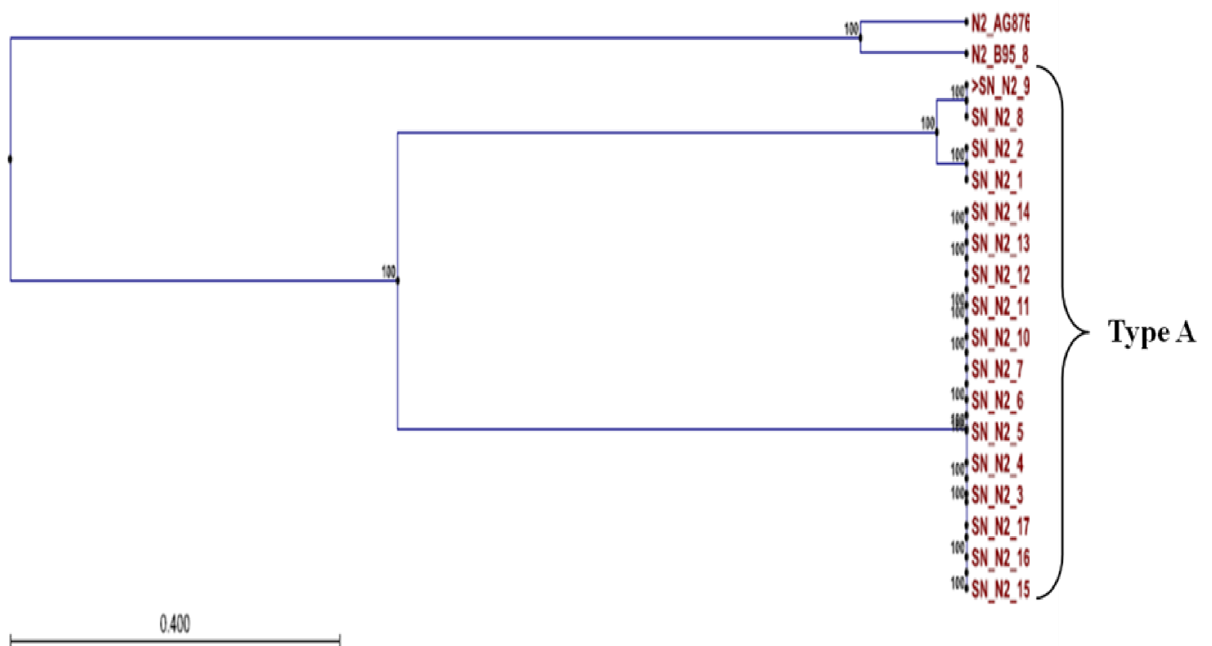
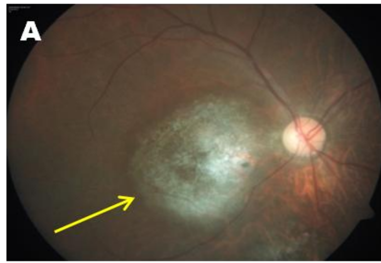
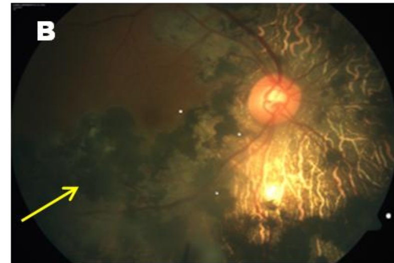


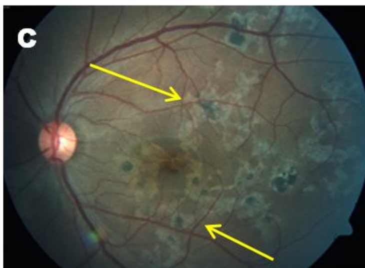
Figure 6.3: Phylogenetic analyses of the EBNA2 PCR positive samples. The 378bp of EBNA2 gene of the representative samples were analyzed by constructing phylogram using UPGMA algorithm together with EBV type A and EBV type B standard strain sequences.



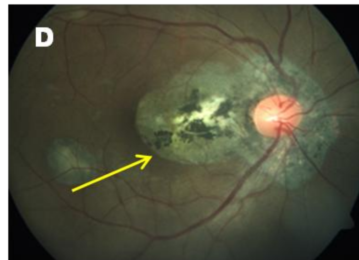
Patient 17: Funduscopy of the left eye at presentation, showing Epstein Barr viral retinitis showing the typical chorioretinal lesions with EBV high titre.



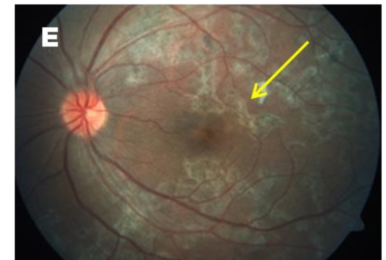
Patient 10: Funduscopy of the left eye at presentation with Giant Helicoid Peripapillary Choroidopathy



Patient 5: Funduscopy of the left eye at presentation with ampiginous choroiditis



Patient 7: Funduscopy of the left eye at presentation with Multifocal choroiditis



Patient 11: Funduscopy of the left eye at presentation with serpiginous choroiditis

Figure 6.4: Fundus photography of patients with high EBV load.

6.5. Discussion:

Herpesviruses represent some of the most successful viruses in humans, infecting over 90% of humans and persisting for the lifetime of the individuals (Knox et al. 1998; Koizumi et al. 2008). Epstein-Barr virus is a recognized cause of intraocular inflammation and has been implicated as a possible cause of ARN. EBV may be directly or indirectly involved in the pathogenesis of a variety of ocular diseases (Wong et al. 1987).EBV was detected in the necrotic retina of patients receiving immunosuppression (Schaal et al. 2014). EBV and CMV causes severe necrotizing retinitis in patients with the acquired immune deficiency syndrome (AIDS) and other herpes viruses have been implicated in the acute retinal necrosis syndrome (ARN), seen in both the immunocompetent and the immunosuppressed. Diagnosis of intraocular EBV induced PTLD can be made from the vitreous using PCR amplification technique (Demols et al. 2001; Hershberger et al. 2003). In this study, nested PCR method was applied to detect EBV, CMV, VZV, HSV, *M. tuberculosis* and *Toxoplasma gondii* in AH samples collected from HIV positive patients with suspected viral retinitis and patients diagnosed multifocal chorioidítis. PCR was used

for genotyping process because of the ease of the technique. Polymerase chain reaction (PCR)-based methods are used for strain (EBV type-A or B) distinction. In the current study, we showed that intraocular HHV-DNA was detectable over a wide range of HHV-associated uveitis when analysis was performed using the two PCR methods. When positive results were noted, we then used real-time quantitative PCR to examine the viral load using commercially available kits based on TaqMan probe assay. This allowed us to confirm our positive results through the use of two PCR combinations. The finding of high viral loads in the ocular fluids suggests that virus replication takes place in the eye, suggesting a direct pathogenic role in intraocular inflammation. The real time PCR results were 100% correlating with that of conventional PCR.

In the current study we have also standardized PCR targeting varying latency genes of EBV for genotyping and applied on clinical samples that are confirmed EBV PCR positive. The entire samples that were tested are found to be EBV Type A by all 3 PCR's targeting genes that codes for EBNA2, EBNA3C, LMP of EBV. The genotyping PCR results were concordant by all the three sets of primers used for amplification of PCR targeting 3 different latency genes of EBV. Since all the three primer sets have given concordant result any of the primer can be used for the genotyping of the EBV. All the previous studies published have analyzed the viral cause of herpetic retinitis by only PCR and further genotyping have not been done(Short et al. 1997; Correa et al. 2004). To our knowledge, this is the first study involved in both detection and genotyping of EBV in ocular samples.

Several studies have shown discrepancies between frequencies of EBV genotypes in blood samples and cultures collected from malignant patients based on the analysis of certain genes such as EBNA-2, 3A, 3B, and 3C (Murray 2000). Previous studies shows applications of PCR on viral DNA from cell culture isolation of clinical samples, in most of the cases, have revealed only one genotype, usually EBV-A (Correa et al. 2004). In contrast, when PCR was applied directly on to the clinical samples, co-infection with both EBV genotypes was observed. The discrepancies may be attributed to biological differences between genotypes, in which the EBV-A is more efficient than EBV-B in the immortalization of growing B-cells in vitro (Sculley et al., 1990). In attempt to avoid this tendency, the present study used PCR

methodology directly in the same sample collected. Type A strain being prevalent in developed and developing countries with a greater potential of transforming B cells while Type B more prevalent in Africa (van Baarle et al. 2000). In this study the application of genotyping PCR to subtype EBV, revealed the circulation of only one subtype (Type A) in ocular sample. In contrast to some studies, which proposed that in HIV-1 infected individuals there is high prevalence of the EBV type B infection or super infection with both type A and B (Reeves et al., 2005). Our study identified EBV-type A in all cases subjected for the study.

6.6. Conclusion:

Use of PCR assay to examine ocular samples in patients with vial retinitis seems to be clinically useful for detecting infectious antigen DNA. Thus, PCR method is a reliable tool for both diagnosing ocular disorders and further screening of patients for intraocular infections. The application EBV type specific PCR described here serves to be a rapid, reliable and cost effective assay to detect EBV subtypes which can be used as an epidemiological tool for clinical management of this virus disease.

Chapter 7

Novel Immune Regulatory Pathway Associated with Epstein Barr Virus (EBV) and the Recombinant EBV Protein Mediated Inflammation in Retinal Pigment Epithelium (ARPE-19).

7.1: Introduction:

Epstein-Barr virus (EBV) is a ubiquitous human lymphotropic γ herpesvirus that latently infects and immortalizes B cells and persists lifelong in resting memory B cells. Ultimately, latent EBV infection can lead to the development of Burkitt lymphoma (BL), nasopharyngeal carcinoma, Hodgkin disease, posttransplantation lymphoproliferative disease, and immunoblastic lymphoma in immunocompromised patients (Young and Murray 2003). Increasing number of ocular disease entities have been reported to be linked to EBV infection. These entities include oculoglandular syndrome, conjunctivitis, dry eye, keratitis, uveitis, choroiditis, retinitis, papillitis and ophthalmoplegia(Maeda et al. 2009). Understanding the biology of EBV infection in human epithelial cells will provide important insights to the role of EBV infection in the pathogenesis of EBV associated ocular infections like Retinitis, Acute Retinal Necrosis etc. Better understanding of immunological response of Acute Retinal Epithelial Cells (ARPE-19) upon challenging with EBV is required for this.

7. 2. Hypothesis:

EBV type – 1 strain and the EBV recombinant proteins NA and P23 are capable of inducing immune response in retinal epithelium which can potentiate the pathology of EBV associated acute retinal necrosis.

7.3. Materials and methods:

Ethics statement: All the experiments were performed with ARPE-19 cells (NCCS, Pune). The study was approved by the Ethics committee, Vision Research Foundation, Sankara Nethralaya, Chennai, India as per the Helsinki declaration.

7.3.1. Cell culture and treatments:

ARPE-19(ATCC) cells maintained in Advanced DMEM F12 (Gibco, USA), 10% FCS (Hi-media, India). The cells were trypsinised and cultured on 24 well plates. The

plates were incubated at 37°C with 10% CO_2. Once the cells formed a monolayer were further used for inoculated with recombinant EBV proteins (Table 6. 1) and virus produced by B95-8 cell line/ EBV type A strain (NCCS Pune). B95-8 culture is filtered through 0.45 um pore size filter and further centrifuged at 3000 x *g* for 15 minutes and the supernatant concentrated by ultra centrifugation at 25, 000 x *g* for 1.5 h at 18deg C. The virus pellet was suspended in RPMI (RoswellPark Memorial Institute medium, Hi-media, India) and stored in -80deg C until used. For infection the virus suspension was diluted 1:10 with RPMI. At 72 hours post treatment with recombinant proteins and virus, cells were harvested and stored at -80°C until subjected for analysis.

7.3.2. Recombinant Epstein Barr viral proteins (Abcam, UK):

The recombinant viral proteins EBNA (Nuclear antigen) and p23 (Viral Capsid Antigen) at concentration of 10 ng/ml were used for study and both were free of endotoxin (0.005 EU/ml) as determined with the limulus amebocite lysate (LAL) assay.

7.3.3. RNA Extraction and cDNA conversion:

Total RNA was extracted using Qiagen RNAse mini kit and 5μg of total RNA was used for cDNA conversion using QuantiTect Reverse Transcription Kit and oligo-dT primers (Fermentas, USA)

7.3.4. RT PCR for GAPDH:

Reverse Transcriptase PCR on cDNA converted from the RNA extracts of challenged cells targeting GAPDH was done to check for the integrity of the cDNA. PCR cycling conditions were as follows, denaturation at 95°C for 10 minutes, followed by 35 cycles of 94°C for 1 minute, 63°C for 1 minute for GAPDH and 72°C for 1 minute, with a final extension of 72°C for 10 minutes. PCR products were loaded on a 2% agarose gel with 0.5μg/ml ethidium bromide and images were captured. The primers used for amplification were, Forward Primer: GCCAAGGTCATCCATGACAAC and Reverse Primer: GTCCACCACCCTGTTGCTGTA and the expected amplicon size was 470 bp.

7.3.5. Real Time PCR for determining the viral load inoculated with B95-8:

The artus® EBV RG PCR Kit (Qiagen) was used to determine EBV load in the

infected cells by real time PCR. 10µl of the sample (viral DNA extracted from infected cells) was added to 15µl of the master mix along with 1 µl of the internal control (provided in the kit). The sample tubes (DNA from B95-8 filtrate and DNA from ARPE cells infected with B95-8 culture filtrate for 72 hrs) along with the quantification Standards (QS 1-4) were subjected to real time PCR (Rotor-Gene).

7.3.6. Immunofluorescence staining:

Cells were seeded on to 11mm coverslips in 35mm dishes, after treatment of the cells with B95-8 for 72 hrs the cells were washed with PBS, fixed with 4% paraformaldehyde for 10 minutes followed by incubation in 10% FBS + 0.01% Triton X-100 in PBS for 15 minutes. The cells were then probed with primary antibody raised in mouse against Epstein Barr nuclear antigen (Santacruz biotechnology, Heidelberg, Germany) and incubated at room temperature for an hour, followed by washing and staining with FITC-conjugated rabbit anti-mouse (Dako, Denmark). The cells were counter stained with DAPI and cover slips were mounted on glass slides with mounting medium. The fluorescent micrographs were taken with a Zeiss Axiovert microscope at 20X magnification.

7.3.7. Real Time PCR for detection of TLRs:

Real time PCR (QuantiTect SYBR Green PCR Kit) was performed with the cDNA converted from RNA extracts from B95-8 culture filtrate and recombinant viral protein treated cell harvest to determine the changes in expression of TLRs 1-10 and GAPDH compared to the cell control. The reaction mixture was prepared according to manufacturer's instruction and the real-time PCR was carried out as follows, 95^0C for 10 minutes, followed by 35 cycles of 94^0C for 1 minute, 55^0C for 1 minutes for all TLRs and 63^0C for GAPDH and 72^0C for 1 minutes, with a final extension of 72^0C for 10 minutes. After setting appropriate threshold, the ct values were noted. The ct values of the TLR's were normalized with respect to the corresponding GAPDH cycle threshold (ct) values and fold change in gene expression for EBV strain-1, p23 and NA treated cells were calculated with respect to the untreated control.The Real time PCR primers used for quantification of TLR's are mentioned in table 7. 1.

7.3.8. Nitric oxide measurement

The stable end product of NO, nitrite was measured by Griess reagent assay. B95-8 and recombinant protein treated cell culture supernatant were collected at the end of 72 hrs and the assay was performed as per the manufacturer's instruction. To study the Involvement of inducible nitric oxide (iNOS) was confirmed by immuno fluorescence staining.

Gene	Primer sequence
TLR1-F	5'ggtcttgctggtcttaggagagac-3'
TLR1-R	5'-ctgaagtccagctgaccctgtagcttcacg-3'
TLR2-F	5'-ggccagcaaattaccacctgtgtg-3'
TLR2-R	5'-ctgagcctcgtccatgggccactcc-3'
TLR3-F	5'-cgggccagctttcaggaacctg--3'
TLR3-R	5'-ggcatgaattatatatgctgc-3'
TLR4-F	5'-tgcaatggatcaaggaccagaggc-3'
TLR4-R	5'-gtgctgggacaccacaacaatcacc-3'
TLR5-F	5'-cctcatgaccatcctcacagtcac-3'
TLR5-R	5'-ggcttcaaggcaccagccatctc-3'
TLR6-F	5'-ccaagtgaacatatcagttaatactttagggtgc-3'
TLR6-R	5'-ctcagaaaacacggtgtacaaagctg-3'
TLR7-F	5'-ctccctggatctgtacacctgtgag-3’
TLR7-R	5'-ctcccacagagccttttccggagct-3'
TLR8-F	5'-gtcctggggatcaaagagggaagag-3'
TLR8-R	5'-ctcttacagatccgctgccgtagcc-3'
TLR9-F	5'-gcgagatgaggatgccctgccctacg-3'
TLR9-R	5'-ttcggccgtgggtccctggcagaag-3'
TLR10-F	5'-cagaggtcatgatggttggatgg-3'
TLR10-R	5'-gacctagcatcctgagataccagggcag-3'

Table 7. 1: Real time PCR primers used for quantification of TLR’s.

7.3.9. Cytokine Expression Array:

The cell supernatants collected after 72 hrs were subjected to TLR-induced Cytokines: Viral-induced Multi-Analyte ELISArray Kit(Qiagen) to estimate the

levels of 12 anti-viral cytokines TNFα, IL-1B, IL-6, IL- 12, 17-A, IL-8, MCP-1, RANTIS, IP-10, MIG, TARC and IFNα. The experiment was performed according to the manufacturer's instructions.

7.3.10. Cytotoxicity test (CCK-8):

After the treatment of cells with B95-8 and recombinant proteins NA and p23 for 72 hrs, the cell culture medium was removed and the cell viability was detected by CCK-8 kit according to manufacturer's instructions.

7.3.11. Apoptosis assay for detection of apoptic nuclei:

The cells were grown in 11mm coverslips in either 35mm dishes or 12 well plates and treaed with B95-8 and recombinant EBV proteins NA and p23 for 72 hrs. At the end of 72hrs treatment the cells were washed with PBS and fixed with 4% paraformaldehyde for 10 minutes. The apoptotic nuceli was detected by terminal deoxynucleotidyl transferase (TdT)- mediated dUTP nick end-labelling (TUNEL) assay by using a commercial kit (TACS® 2 TdT-Fluor *In Situ* Apoptosis Detection Kit) according to the manufacturer's protocol. In the TUNEL method, the 3'-OH ends of DNA fragments are nick-end labeled with FITC-dUTP (or dUTP-biotin and avidin-FITC); this process is mediated by terminal deoxynucleotidyl transferase (TdT). In brief, the paraformaldehyde fixed cells were rinsed with PBS for 10 minutes, followed by incubation with proteinase K for 15 minutes at room temperature. The cells were incubated with the reaction mix for 60 minutes in a humidity chamber at 37^{0}C followed by Strep-Flour labelling. The coverslips were mounted on to a glass slide and fluorescent images were captured by Zeiss Axiovert microscope.

7.3.12. Detection of stages of apoptosis in treated cells:

The FACS assay was performed using the FITC Annexin V Apoptosis Detection Kit II (BD Pharmingen™). The cells were grown in 6 well Tissue culture plate and treated with B95-8, p23 and NA. After 72 hrs of treatment the cells were washed with PBS. The cells were then spun at 2000 rpm for 5 minutes and then resuspended in 200 µl of Binding Buffer. 2.5 µl of PI (Propidium Iodide) and 3 µl of Annexin V were added to the cells and the cells were then incubated at 4°C for 1 hour in the

dark. The cells were washed twice with 1X Binding Buffer and were then analyzed by flow cytometry.

7.3.13. Processing of Virus infected ARPE – 19 cells for Electron Microscopy:

After 72 hrs of virus treatment the cells were harvested and washed with PBS. The cells are pelleted by centrifugation at 30, 000*g* for 2hrs and the virus infected cells are fixed with 2.5% gluteraldehyde in PBS for 20 min at 4°C. The cell pellet was replaced with PBS after 4 hrs of fixation and cells are post-fixed with 1% osmium tetroxide in the above buffer at 4°C for 1 hour. Cells dehydrated in a graded series of ethanol or acetone and resuspended in 100% acetone, with pelleting at 2000 RPM. Cells resuspended in a 50:50 mix of acetone: embedding medium LX112 resin mix and pelleted at 3000 RPM, and resuspend them in 100% embedding mix and infiltrated cells in embedding mix for 1 hour under vacuum at 7800 RPM for 15 min. Cells are polymerized in embedding mix at 70°C oven overnight and fixed on trimming block and ultra thin sections were taken and carried on copper grids. Grids stained with 8% aqueous uranyl acetate, and examined with Jeol Jem 1400 TEM.

7.4. Results:

7.4.1. EBV recombinant proteins induced morphological changes in ARPE cells:

The ARPE cells were treated with EBNA, P23 and Type-A strain of the virus (B95- 8) along with cell control and incubated at 37°C for 72 hrs. The cell control showed 100% confluency (Figure 7.1A). Morphological effects were most visible in the cells treated by EBNA and p23 where plaques were visible under phase contrast microscope (figure 7.1B, 7.1C). Morphological changes such as plaques, in cells treated with p23 and NA signifying cellular changes induced by antigenicity of both recombinant viral proteins. All treated cells had become granulated and had formed clusters.

7.4.2. EBV infection and proliferation in ARPE cells: B95-8 infected retinal cells showed cytopathic changes such as enlargement of the cells, increased nuclear cytoplasmic ratio, and plaques after 72 hrs (figure 7.1D). EBV RNA level increased from 8.73 copies/µl on day 0 to 28.54 copies/µl on day 3 (Figure 7.2A). Viral NA expression was detected in B95-8 infected retinal cells by immunofluorescence (Figure 7.2B).

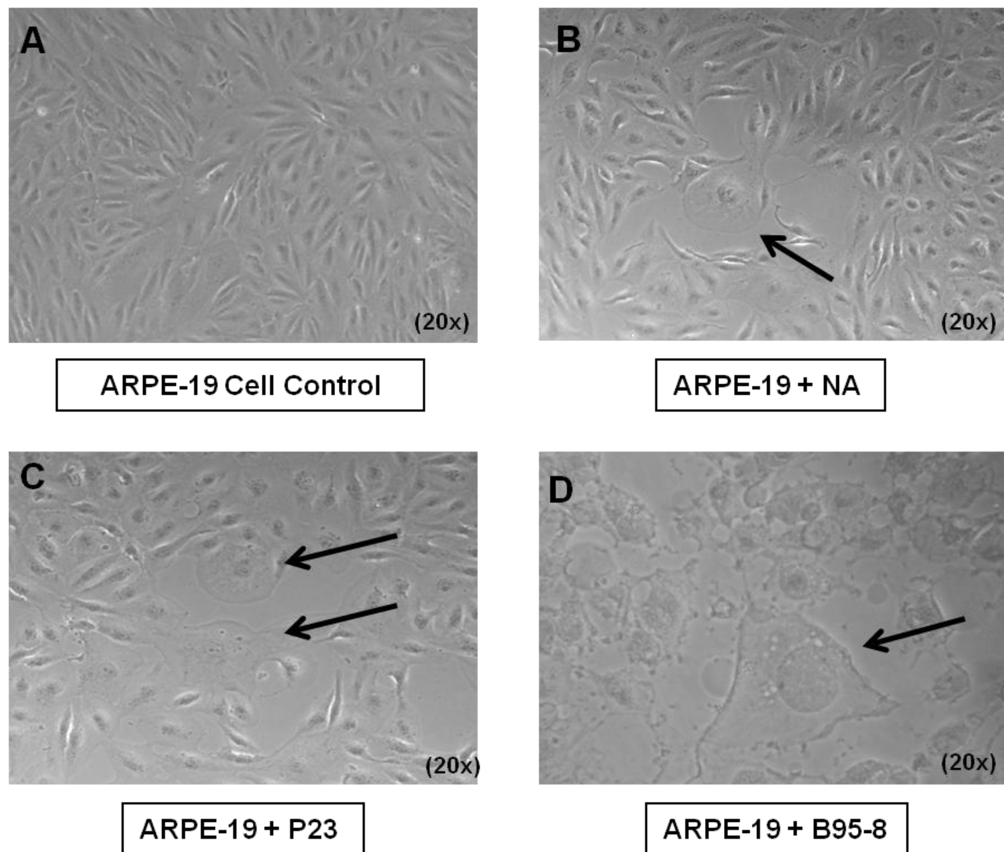


Figure 7.1: Cells observed under phase contrast microscope at 72 hours post treatment with recombinant proteins and EBV Type A strain – B95-8.

The ARPE cells were treated with EBNA, P23 and Type-I strain of the virus (B95- 8) along with cell control and incubated at 37°C for 72 hrs.

- The cell control showed 100% confluency (Figure 7.1A).
- Morphological effects were most visible in the cells treated by NA and p23 where plaques were the cells enlarged in size, cytoplasmic blebbing and plaques were observed visible under phase contrast microscope (Figure 7.1B and 7.1C).
- All treated cells enlarged in size and observed with cytoplasmic vacuoles and lost their morphology and granulated and had formed clusters and plaques observed in B95-8 fitrate infected ARPE-19 cells (figure 7.1D).

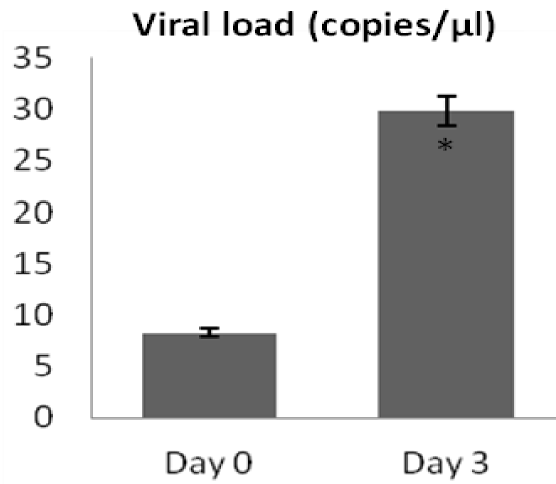


Figure 7.2A: Real time PCR for detection of Epstein Barr viral load:

EBV real time PCR performed on 72 hrs treated cell culture harvest showed increase in EBV viral RNA from 8.73 copies/µl on day 0 to 28.54 copies/µl on day 3 (Figure 7.2 A).

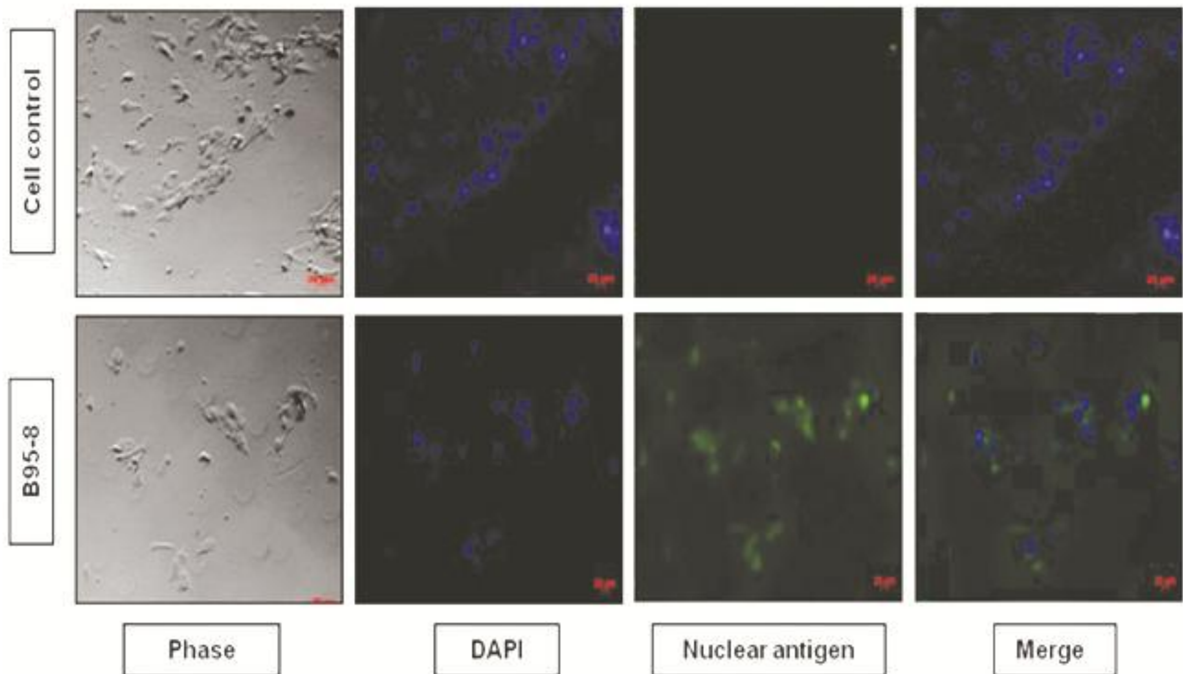


Figure 7.2B: Immunofluorescence staining targeting Epstein Barr nuclear antigen:

Immunofluorescence staining performed on cell control and viral treated cells showed the expression of Epstein Barr Nuclear antigen in the B95-8 treated cells (Figure 7.2B).

7.4.3. RT PCR for GAPDH:

The RT PCR products were run on a gel and GAPDH product corresponding to the 470 bp was detected. The presence of GAPDH bands indicates that the extracted RNA has not disintegrated. (Figure 7.3)

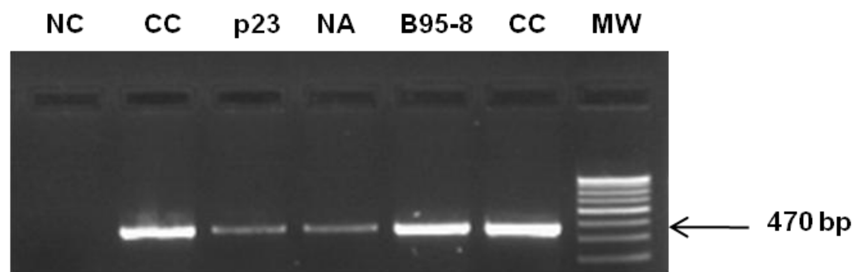


Figure 7.3: Agarose gel electrophoretogram showing amplification of GAPDH gene in the RNA extracted from Epstein Barr recombinant proteins and type A EBV treated ARPE cells. NC: Negative control, CC: Cell control, p23: RNA extracted from p23 (10ng/ml) treated ARPE-19 cell culture harvest, NA: RNA extracted from Nuclear antigen (10ng/ml) treated ARPE-19 cell culture harvest, B95-8: RNA extracted from EBV type A strain, B95-8 culture filtrate infected Cell harvest, MW: 100bp ladder.

7.4.4. Involvement of toll like receptors in the innate immune response in ARPE against EBV p23 and NA proteins and B95-8:

We hypothesized the involvement of toll like receptors (TLRs) in EBV p23 and NA and B95-8 mediated inflammatory response. Real Time PCR was performed to measure the gene expression and to detect the involvement of 10 TLRs in mediating inflammatory response. The up regulation and down regulation of TLR genes were calculated by performing Real Time PCR with equal amount of cDNA for control, protein and B95-8 treated samples. All the TLR Cycle Threshold (ct) values were normalized with respect to the corresponding GAPDH values and TLR gene up regulation or down regulation were calculated with respect to the untreated control cells.

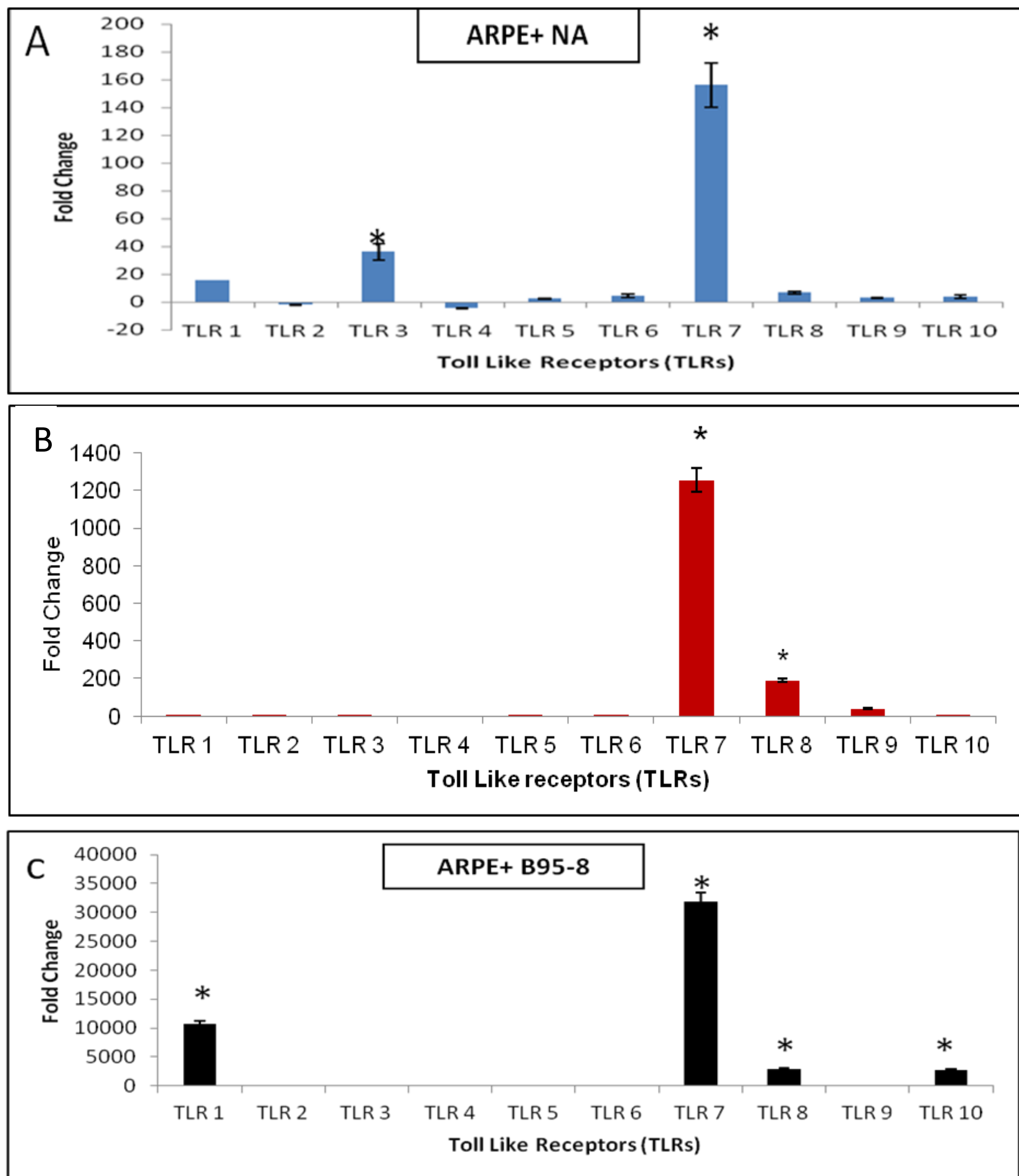


Figure 7.4: EBV recombinant proteins and EBV type A strain induced toll-like receptor activation. Epstein Barr virus recombinant proteins NA, p23 and B95-8 culture filtrate induced toll-like receptor activation. ARPE-19 cells were exposed to B95-8 filtrate and 10 ng/ml of NA and p23 proteins for 72 h. Real time PCR was performed to measure the *TLR* gene expression. Upon exposure to NA, TLR1, 3 and 7 were significantly up regulated; TLR2 and 4 were significantly down regulated (Figure 7.4A). During the p23 protein treatment TLR7 and TLR8 were significantly upregulated (Figure 7.4B). There was drastic increase in fold change cells in the cells infected with B95-8 (Figure 7.4C). Apart from TLR 7, TLR 8, TLR 1 and TLR 10 were also significantly upregulated in B95-8 filtrate treated cells.

7.4.5. Human retinal epithelial cells released cytokines upon exposure to Virus and recombinant protein:

Human retinal epithelial cells were exposed to B95-8 and viral proteins for 72 hrs and ELISA array were performed to detect cytokines in the cell culture supernatant.Treatment of ARPE-19 cells with NA and p23 has caused significant over expression of IL-6, IL-8 and MCP 1 (Figure 7.5A). Infection of B95-8 has caused increased expression of MIG, IP-10, IL-1β, TNFα, RANTIS and IL-17A. IFNα was also highly secreted in the virus infected and recombinant protein treated cell culture supernatants (Figure 7.5B).

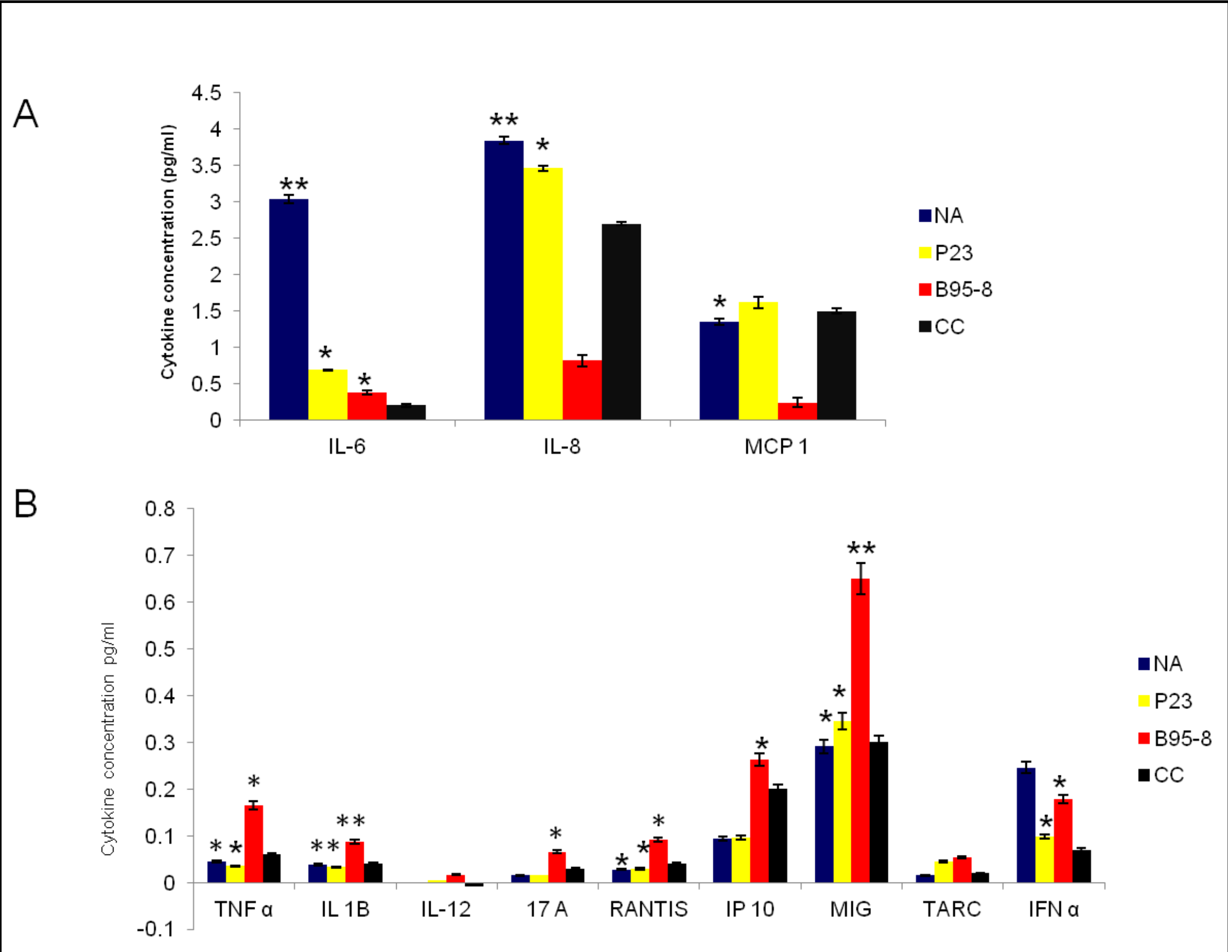


Figure 7.5: Cytokines response upon exposure to Virus and recombinant protein.

Epstein Barr virus NA and p23-induced cytokine production. ARPE-19 cells were exposed to 10 ng/ml of NA and p23 proteins for 72 h. Enzyme-linked immunosorbent assay (ELISA array) was performed to detect cytokines in the cell culture supernatants. A,B: Core and NS3 induced interleukin (IL)-6, IL-8, secretion at a highly significant level and TNFα, IL-1 β, RANTIS, MIG to a significant level. B95-8 has caused increased expression of TNFα, IL-1β,IL-17A, RANTIS, IP-10, MIG. Data are represented as mean ± standard error of the mean (SEM) over the untreated control group. *p<0.05, ** p<0.001.

7.4.6. Cell signaling mechanism involved in TLR mediated immune response:

MyD88 being an important adaptor protein involved in TLR mediated immune activation we suspected the involvement of MyD88 in EBV p23 and NA and B95-8 mediated immune response in ARPE. Seventy two hrs of exposure to EBV p23, NA protein and B95-8 triggered up regulated expression of MyD88 in ARPE (Figure 7.6A). Immunofluorescence staining was performed for MyD88 and nuclear translocation factor-kappa B (NF-κB) Fluorescent micrographs show that MyD88 protein expression was up regulated in the p23, NA protein and B95-8 exposed cells at the 72 h time point, but the same was not detected at the 24 h time point (Figure 7.6B). We could not detect NF-κB nuclear translocation at the 24 and 72h time points (Figure 7.6C).

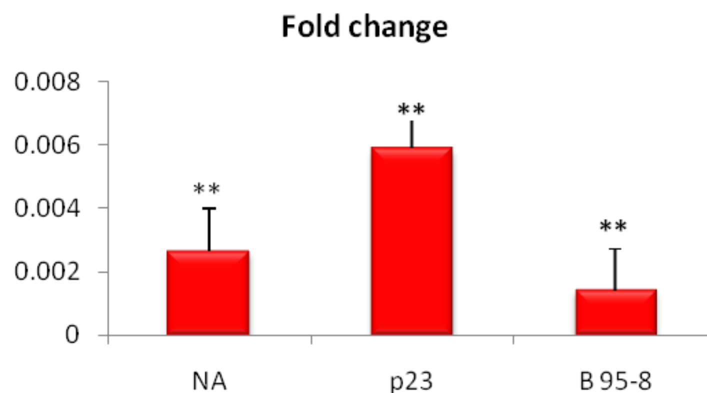


Figure 7.6A: Real time PCR for MYD88: EBV p23 and NA and B95-8 induced upregulated expression of MyD88. ARPE-19 cells were exposed to 10 ng/ml of p23 and NA proteins. Semi quantitative reverse transcription (RT)–PCR was performed to measure the *MyD88* gene expression. Data are representative of three independent experiments. Data are represented as mean ± standard error of the mean (SEM) over the untreated control group. *p<0.05, **p<0.01.

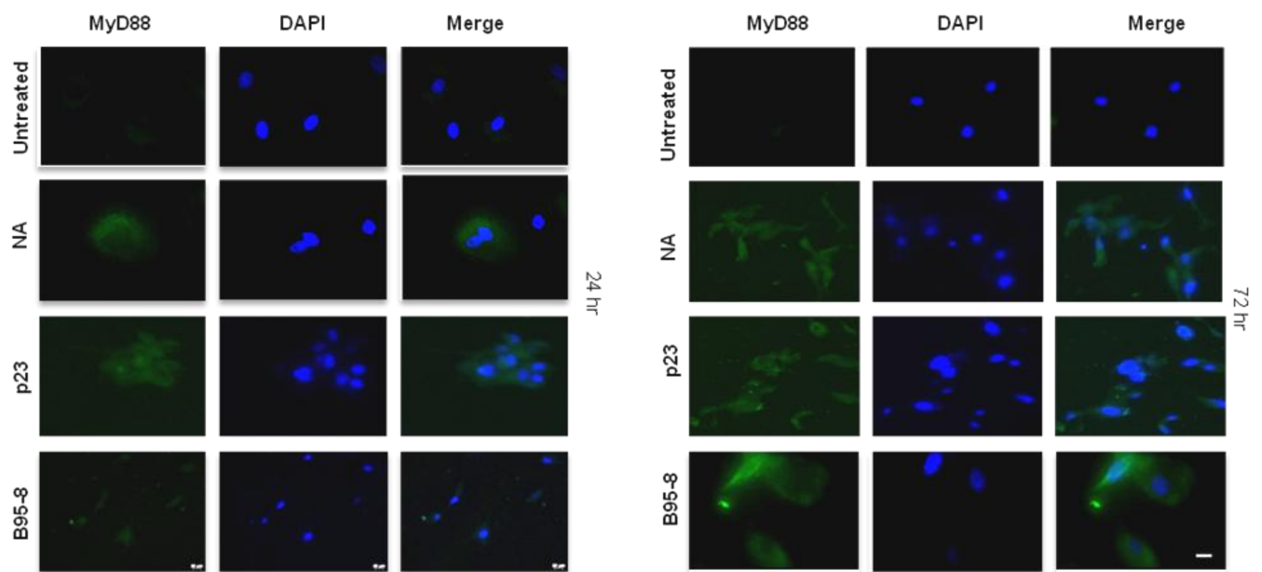


Figure 7.6B: Immunofluorescence staining for MYD88:Toll-like receptors signaled via MyD88. ARPE-19 cells were exposed to 10 ng/ml of NA and p23 proteins and B95-8 filterate for 24 h and 72 h. Immunofluorescence staining was performed for MyD88 and nuclear factor-kappa B (NF-κB). At the 72 h time point, MyD88 fluorescent intensity increased in the NA and p23 proteins and B95-8 filterate treated cells compared to the untreated control. At the 24 h time point, the fluorescent signal for NA and p23 proteins and B95-8 filterate treated cells was similar to that of the control cells. Scale bar=20 µm.

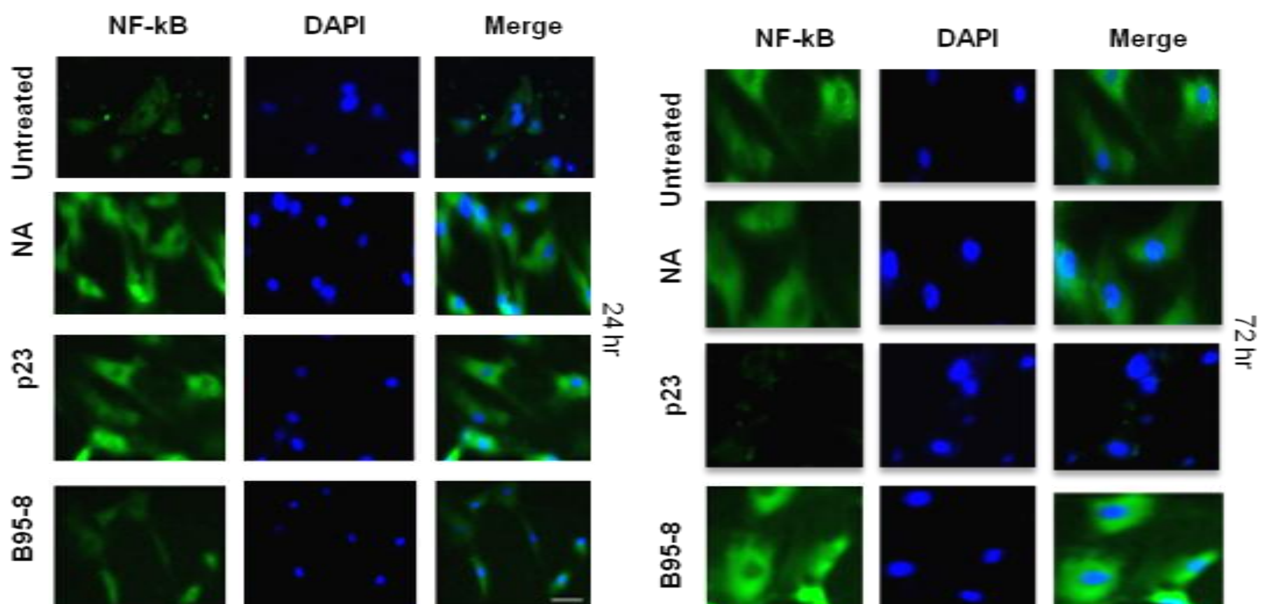


Figure 7.6C: Immunofluorescence staining for NF-κB. ARPE-19 cells was exposed to 10 ng/ml of NA and p23 proteins and B95-8 filtrate for 24 h and 72 h. Immunofluorescence staining was performed for nuclear factor-kappa B (NF-κB). At the 24 h and 72 h time points, NF-κB nuclear translocation was not observed for the NA and p23 proteins and B95-8 filtrate treated cells. Data are representative of three independent experiments. Scale bar= 50 µm

Nitrite, a stable and non-volatile product of Nitric Oxide (NO) was measured in the culture supernatants exposed to virus and viral recombinant proteins at 72 hrs by using Griess's reagent. NO production was detected at highly significant level in the culture supernatants exposed to B95-8 filtrate and significant level of NO was detected in culture supernatant in p23 and NA treated ARPE-19 cells (Figure 7.7A). Involvement of inducible nitric oxide (iNOS) was confirmed by immuno fluorescence staining. iNOs was expressed in ARPE cells exposed to NA, p23 and B95-8 filtrate (Figure 7.7B).

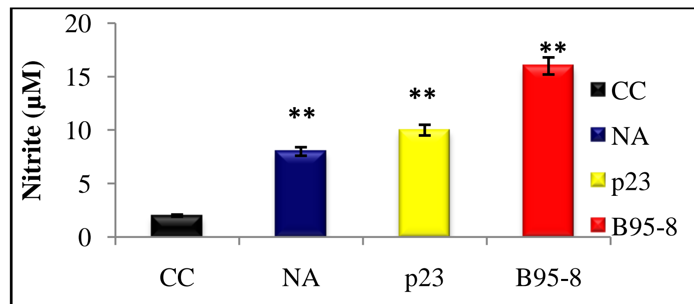


Figure 7.7A: Nitric oxide (NO) production detected in treated and untreated cell culture supernatants. ARPE-19 cells were exposed to 10 ng/ml of NA and p23 proteins and B95-8 filtrate for 72 h. NA, p23 protein and B95-8 filtrate exposed cells synthesized nitric oxide (NO) at 72 h. Data are represented as mean ± standard error of the mean (SEM) over the untreated control group. *p<0.05, **p<0.01.

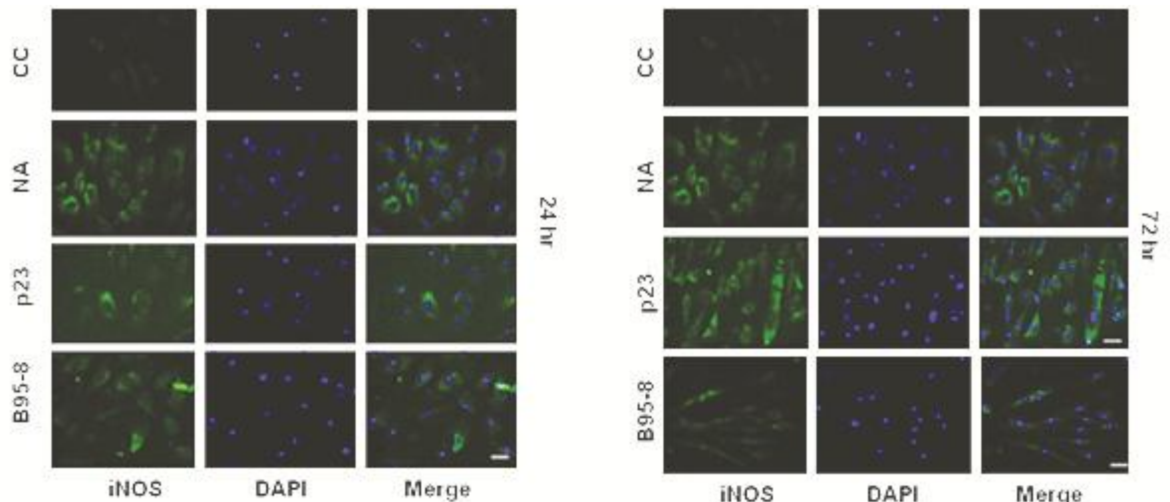


Figure 7.7B: iNOS expression detected by immunofluorescence staining.iNOS protein fluorescent intensities were comparatively high in the NA and p23and B95-8 treated cells at 24and 72 h. Data are representative of three independent experiments. Scale bar = 50 µM.

7.4.8. EBV NA, P23 and B95-8 mediated apoptosis and cell death in ARPE-19:

TUNEL assay was performed to detect the apoptic cells at 72 hrs exposure toproteins and B95-8. Retinal cells underwent apoptosis within 72 hrs exposure to NA. Increase in apoptic cells were observed in p23 treated cells when compared with NA. Almost all the cells treated with B95-8 showed fluorescence signifying increased percentage of retinal cells apoptosis (Figure 7.8A). The cell viability was measured by Cell Counting kit (CCK-8) assay. At 72 hrs time point when compared to the untreated control group, there was significant difference in cell viability for p23 (35%) treated cells when compared to NA treated cells (41%) signifying the antigenic property of p23 which involves lytic activation of EBV *in vivo*. Cell viability was profoundly decreased in B95-8 cells (24%) when compared with cells treated with recombinant viral proteins. (Figure 7. 8B).

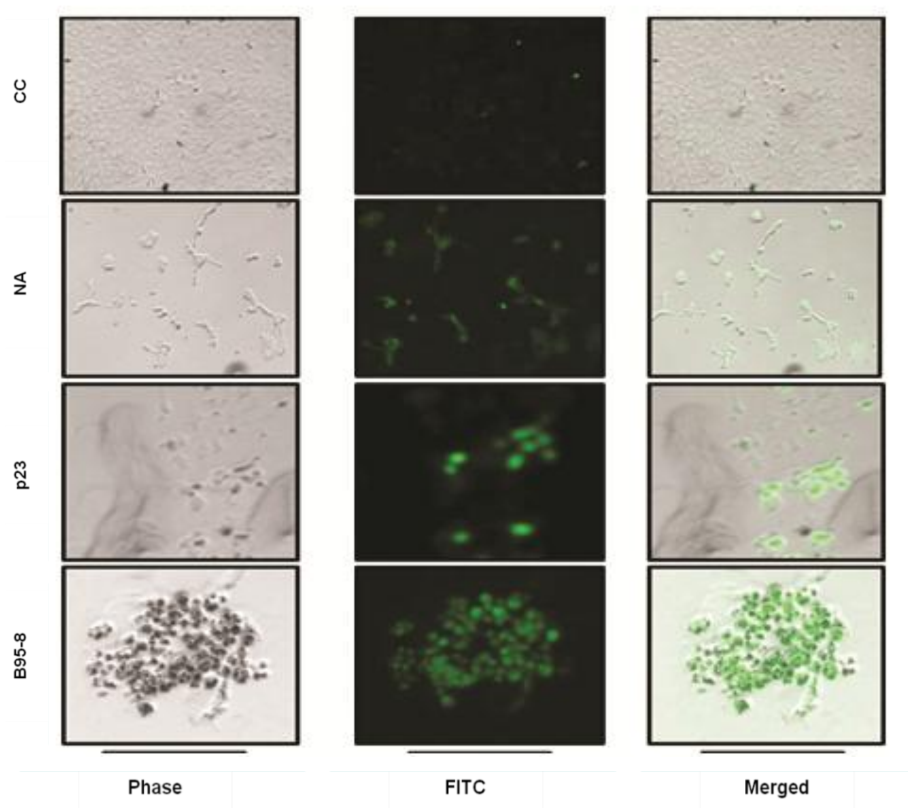


Figure 7.8A: TUNNEL assay for the detection of apoptosis. The terminal deoxynucleotidyl transferase-mediated uridine 5′-triphosphate-biotin nick end labeling (TUNEL) assay was performed to detect apoptosis. ARPE-19 cells were exposed to 10 ng/ml of NA and p23 proteins and B95-8 filtrate for 72 h. Cells stained positive for apoptotic nuclei during the NA and p23 treatment, and this were absent in the control cells. The number of apoptotic cells in the B95-8 treated ARPE cells was significantly higher than that in the recombinant protein treated ARPE-19 cells. Data are representative of three independent experiments. Scale bar=50 μm.

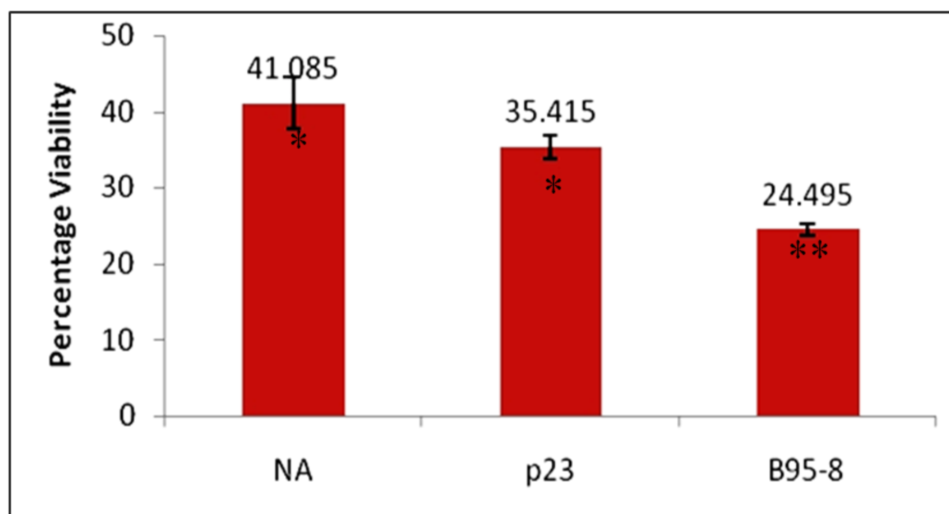
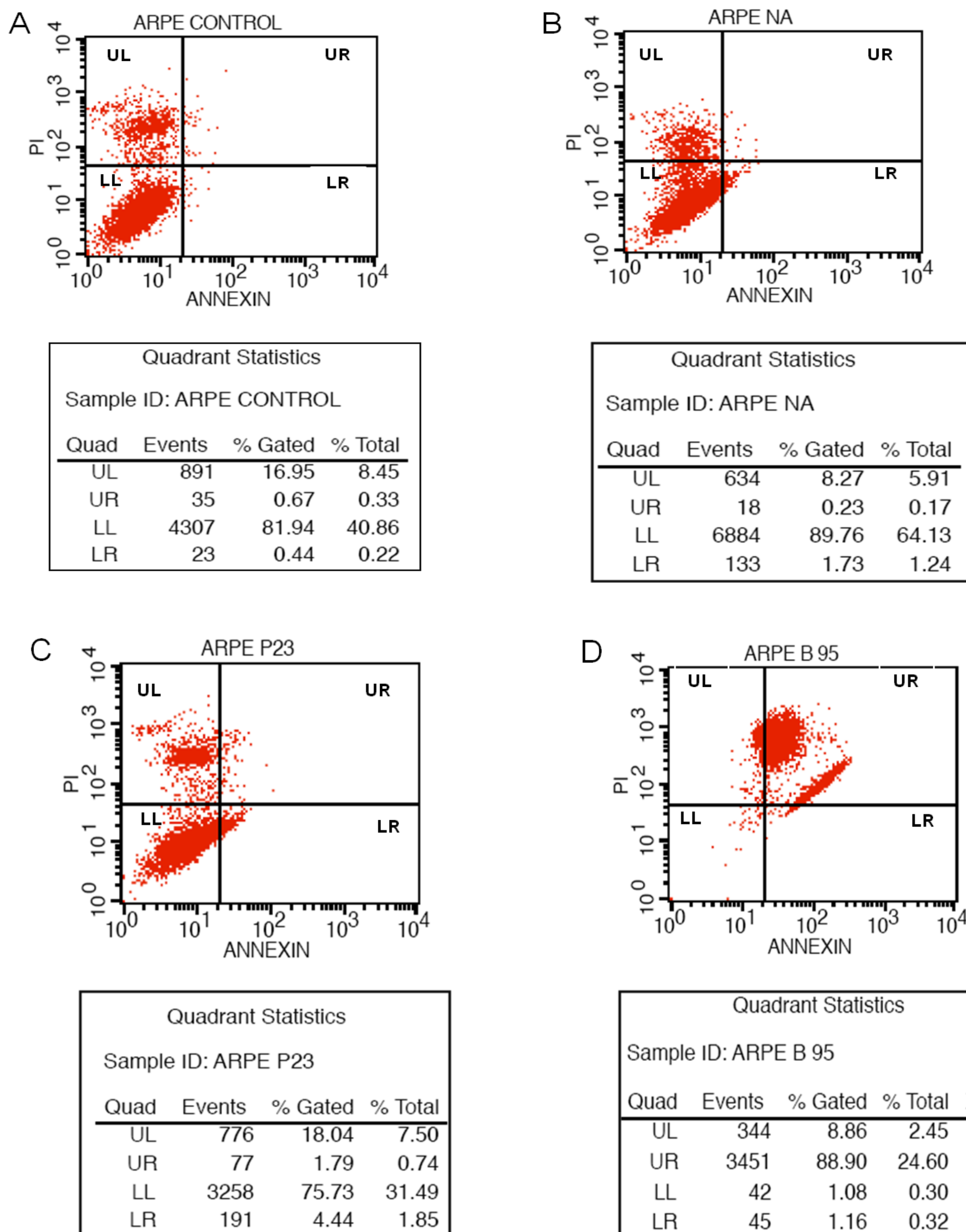


Figure 7.8B: Percentage Viability of cells infected with NA,p23 and B958 compared to cell control.The water-soluble tetrazolium salt WST-8[2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium,monosodium salt], CCK-8 assay (using cell counting kit - 8) was performed to measure cell viability. EBV recombinant proteins NA, p23 and B95-8 filtrate induced apoptosis and cell death. ARPE-19 cells were exposed to 10 ng/ml of NA and p23 proteins and B95-8 filtrate for 72 h. During the NA and p23 proteins and B95-8 filtrate treatment, cell viability significantly decreased at 72 h. Data are represented as mean ± standard error of the mean (SEM) over the untreated control group. *$p<0.05$, **$p<0.01$.

Flow cytometry analysis of ARPE-19 cells treated with NA, p23 and B95-8 filtrate for 72 h revealed that 8.27% of the cells underwent necrosis treated with NA (Figure 7.9B), while there was increase in percentage of cells (18.04%) undergone necrosis in p23 treated cells (figure 7.9C) and there was a drastic increase in the percentage (88.9%) of cells treated with B95-8 filtrate in end stage of apoptosis or dead cells and 8.86% of cells underwent necrosis (figure 7.9D).



Quadrant Statistics

Sample ID: ARPE CONTROL

Quad	Events	% Gated	% Total
UL	891	16.95	8.45
UR	35	0.67	0.33
LL	4307	81.94	40.86
LR	23	0.44	0.22

Quadrant Statistics

Sample ID: ARPE NA

Quad	Events	% Gated	% Total
UL	634	8.27	5.91
UR	18	0.23	0.17
LL	6884	89.76	64.13
LR	133	1.73	1.24

Quadrant Statistics

Sample ID: ARPE P23

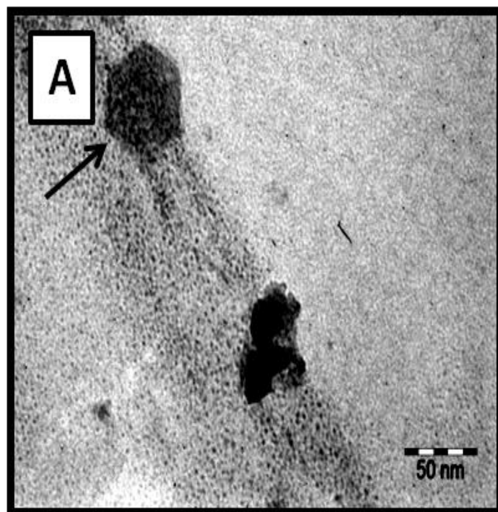
Quad	Events	% Gated	% Total
UL	776	18.04	7.50
UR	77	1.79	0.74
LL	3258	75.73	31.49
LR	191	4.44	1.85

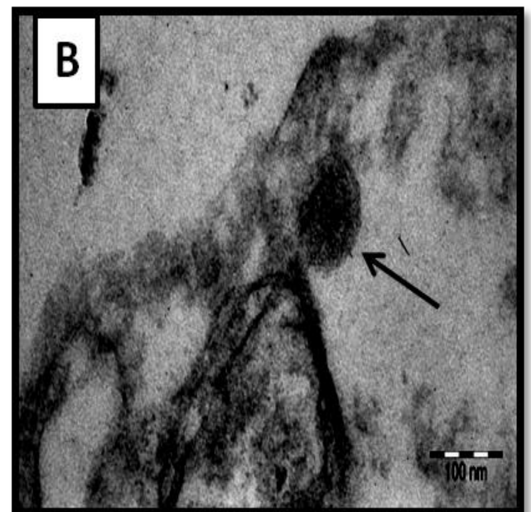
Quadrant Statistics

Sample ID: ARPE B 95

Quad	Events	% Gated	% Total	X
UL	344	8.86	2.45	
UR	3451	88.90	24.60	
LL	42	1.08	0.30	
LR	45	1.16	0.32	

Figure 7.9. Percentage Viability of cells infected with NA, p23 and B958 compared to cell control**:** Flow cytometry analysis of arpe-19 cells treated with NA, p23 and B95-8 filtrate for 72 h. Treated cells were examined for apoptotic cells using Annexin V-FITC apoptosis detection kit. A: Both Annexin Vand PI-negative cells were viable cells, B:Annexin V-positive/PI-negative cells were in early stages of apoptosis and C: double positive cells were in late apoptosis, whereas D: annexin V- negative/PI -positive cells were necrotic.





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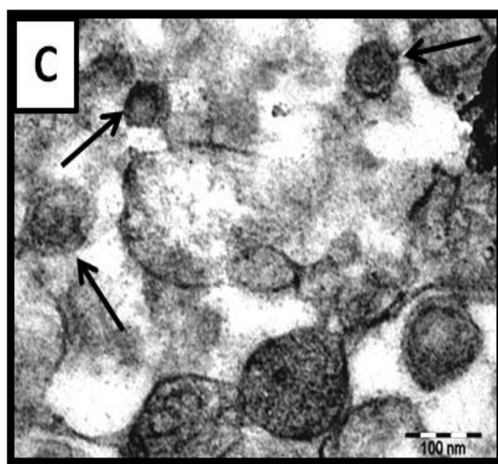

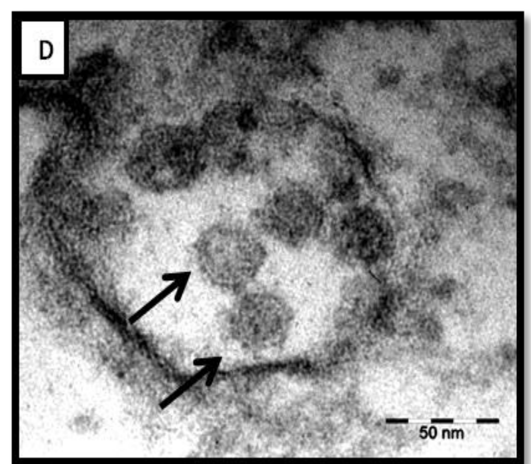

Figure 7.10. Electron micrographs of Epstein Barr virus in the infected ARPE-19 cells: A and B: Single mature enveloped particle with central, dark staining nucleic acid (DNA) at the cell membrane of infected cell, C and D : Numerous mature and imature particles lying in the cytoplasm of infected ARPE-19 cells.

7. 5. Discussion:

Host resistance to EBV infections includes nonspecific mechanisms involving IFNs, complement, macrophages, humoral (antibody) immunity, T cell-mediated immunity (such as cytotoxic T cells (CTLs) and T helper cell activity), and cytokine release. Animal studies have suggested that activated macrophages, IFNs and to a lesser extent, natural killer cells are important in limiting initial EBV infection, whereas humoral immunity and cell-mediated immunity are important in controlling both initial and recurrent infections (Taro Kawai et al. 2009). In this context we examined the inflammatory potential of two major EBV antigens, NA and p23 and EBV Type A strain (B95-8) in mediating inflammation and the associated innate immune response via TLR's

in ARPE cells. Morphological changes were observed in the cells treated with NA and p23. The cytopathic effects are observed in cells infected with B95-8 filtrate.

The Real Time PCR analysis for TLR expression levels in cells treated with B95-8 showed an up regulation in TLR 7. TLR 7 is commonly up regulated in response to of viral genomes (Hemmi et al. 2002). Similarly in cells treated with NA and p23 also the level of TLR 7 is up regulated. Up regulation of TLRs in cells infected with the viral strain is highly significant when compared to the up regulation in cells treated with recombinant viral proteins. In our experimental condition EBV NA and p23 proteins were not found to be very specific for single TLR as there was difference in the expression patterns of multiple TLRs in both the condition followed by up regulation and down regulation of multiple TLRs. The up regulated response of TLR transcripts can be explained by the innate immune mechanisms to eradicate the pathogen by mediating the release of pro inflammatory cytokines and the down regulation of some of the TLRs could be a mechanism of immune evasion mediated by the EBV proteins (Janssens and Beyaert 2003). This is the first ever demonstration of TLR response in retinal cells by EBV viral proteins which are found to be bifunctional as we can see the expression IL-6, which actions as anti-inflammatory along with IL-8 secretion. We could observe MyD88 signalling evidenced by IF staining (Figure 6A) and by expression of MyD88 at 72 hrs post treatment (Figure 6B). MyD88 being a normal adaptor protein associated with TLR signalling we could definitely appreciate the involvement of TLRs in innate immune response, without involving NF-κB in the signalling mechanism which contributed to the cytokine release and ultimately inflammation. Our results are in distinction to the common TLR signalling via NF-κB. In this particular condition there might be other transcription factors such as IRF-7, IRAK4, TRAF6 which might have involved in the TLR signalling via MyD88 in activating the pro-inflammatory cytokine genes (Honda et al. 2004). This study shows that various TLR’s responds via MyD88 in ARPE.

The cytokine expression array (ELISA) showed that IL-6, IL-8 and MCP 1 are up regulated in response to recombinant viral protein treatment and viral infection when compared to cell control. IL-6 is responsible for inhibitory effects on TNF-alpha and IL-1, and activation of IL-1 and IL-10. IL-8 is responsible for chemotaxis in target cells and also induction of phagocytosis. The role of IL-8 in ocular inflammations is found to be bifunctional which is evidenced by the participation in neovascularisation and wound healing (Strieter et al. 1992, Strieter et al. 1995). Role of IL-6 in ocular inflammation is

found to be pro-inflammatory, as this cytokine was promoted virus associated corneal inflammation followed by leukocyte infiltration and along with IL-8 and MCP results in cell apoptosis (Matsukawa et al. 1999). Hence these TLR induced cytokines are over expressed in the cells treated with viral proteins and EBV type A strain. MIG, RANTIS, IP 10 and IL17A are significantly up regulated in cells infected with B95-8 which in turn induces IFNα. IFN α is mainly involved in innate immune response against viral infection and involved in cell adhesion, chemo attractant which can induce immune cell migration to the site of inflammation and wound healing. Hence the properties of these cytokines can be correlated with their up regulation after viral treatment followed by involvement of inducible nitric oxide (iNOS) was confirmed by immuno fluorescence staining. iNOs was expressed in cells exposed to B95-8 leading to significant levels of measurement of nitric oxide.

The increase in viral load detected by real time PCR indicates that the virus proliferation was significant in the infected ARPE-19 cells. Electron microscopic examination revealed that the virus is capable of infecting and proliferating in ARPE-19 cells inferred by mature enveloped viral particle at the cell membrane of infected cell and numerous mature and immature particles lying in the cytoplasm of infected ARPE – 19 cells.

Apoptosis is a highly programmed cell death followed by distinctive biochemical events. It is found to be the end stage in many inflammatory conditions. IL-8 and NO is known mediators of apoptosis. We tried to find out whether EBV antigenic proteins could mediate apoptosis in Retinal cells. The reduction in the percentage of viable cells detected by CCK-8 assay indicates that the cells treated with both recombinant viral proteins and virus were underwent either cell death or apoptosis. These results were further confirmed by performing FITC Annexin-V and propidium iodide staining apoptosis assay and TUNNEL assay. The cells treated with NA and p23 have undergone necrosis and 89% of cells infected with B95-8 were undergone later stage of cell death. The percentage of cell death is highest in cells infected with B95-8 followed by p23 and then NA. Based on these results we hypothesize a pathway by which EBV proliferation in retinal cells can mediate inflammation in retinal cells, which in turn, can form pathogenesis of retinal necrosis in patients with chronic EBV infection (Figure 7.11).

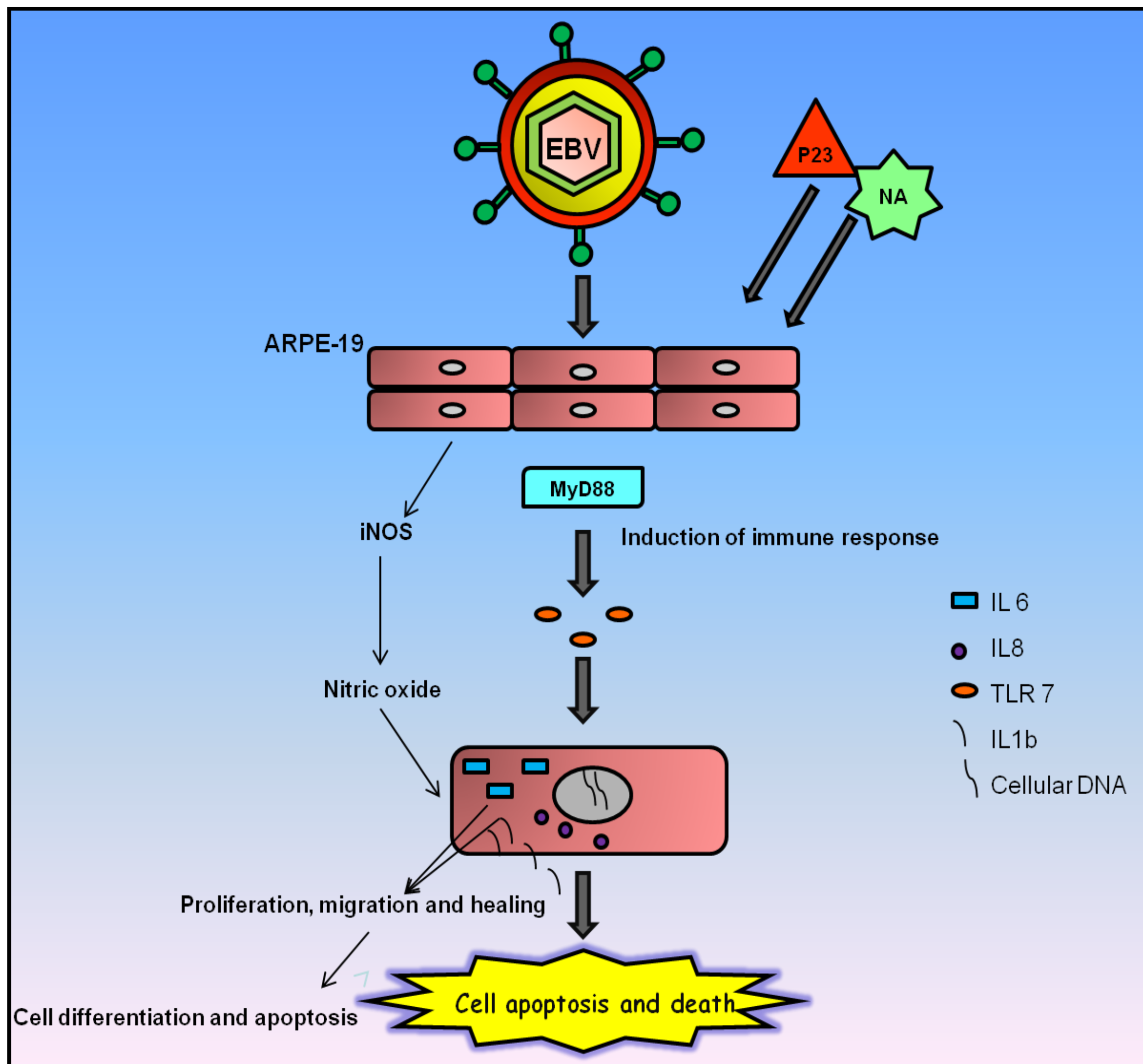

Figure 7.11. Schematic representation of a possible inflammatory pathway: The toll-like receptor (TLR) ligands identify NA and p23 proteins, and MyD88 transfers the signals to transcription factors, which, in turn, induce cytokine gene expression. Adult retinal epithelial cells synthesize nitric oxide (NO) via inducible nitric oxide synthase (iNOS). These stress responses induce apoptosis and cell death.

7.6. Conclusion:

To the best of our knowledge this is the first time an *in vitro* study was being carried out to detect the crucial roles in ARPE signalling in response to EBV viral proteins p23 and NA; and EBV Type A strain (B95-8). These findings may provide crucial information for understanding the immune mechanisms of EBV induced inflammation and cell death in ARPE and help design new immune therapeutical approaches to ocular infections caused by EBV.

8. OVER ALL CONCLUSIONS

PCR targeting *VCA* and *EBNA1*genes for detection of Epstein Barr Virus were standardized and applied on to clinical samples collected from patients suspected with Infectious mononucleosis, pediatric post liver and kidney transplant patients and patients with viral retinitis.VCA PCR was found to be highly sensitive for detection of early primary infection.PCR based detection of infectious mononucleosis ruled out cross reactivity caused by other infectious agents like Human cytomegalovirus.

PCRtargeting *EBNA2* and *EBNA3c*genes of EBV were standardized for genotypic detection of EBV in samples collected from pediatric post transplant patients and ocular samples collected from patients suspected with viral retinitis.Genotyping PCR revealed circulation of both genotypes A and B in post transplant immunocompromised patients, predominantly Type A and circulation of only EBV Type A in ocular infection and also in healthy control population. This is the first time EBV genotyping based study is carried out in India. Although disease-specific genotypes or strains were not identified, this study provides useful insights into the molecular epidemiology of EBV infection in India.

Detection of EBV RNA and viral load in samples obtained from pediatric post liver transplant and patients with suspected infectious mononucleosis revealed thatEBV RNA levels were significantly higher during post transplant patients than IM and the presence of viral RNA implies that active transcription occurs. EBV viral load was not detected in control population. Levels of IL-6 and IL-17a were significantly higher at high EBV titre levels observed during Post transplant Lymphoproliferative disorder and acute infectious mononucleosis. This study clearly demonstrates the significance of active EBV infection (RNA expression) in key cytokine production and demonstrates the important role of IL-6 and IL-17a during acute EBV infection and suggests that these cytokines together with anti-EBV antibody control EBV proliferation during acute EBV infection in IM and post transplant patients and likely preventing PTLD. This finding opens novel avenues to study cellular and/or viral gene expression and will introduce new biomarkers and strategies to improve clinical as well as diagnostic practices in chronic EBV infection.

In- vitro study performed to determine the molecular mechanism by which EBV induces inflammation in the retinal epithelial cells revealed that EBV is capable of infecting and proliferates in adult retinal epithelial cells (ARPE-19) leading to necrosis and cell death.

EBV type A strain and Epstein Barr viral recombinant proteins, latent protein - Nuclear antigen and lytic protein - p23 induced morphological changes in ARPE cells and induced inflammation in ARPE via toll like receptor mediator signaling. Human retinal epithelial cells released cytokines upon exposure to EBV type A and recombinant viral proteins and produced nitric oxide via inducible nitric oxide synthase leading to apoptosis and cell death. These findings provide crucial information for understanding the immune mechanisms of EBV induced inflammation and cell death in ARPE and help design new immune therapeutically approaches to ocular infections caused by EBV.

9. SPECIFIC CONTRIBUTIONS OF THE PRESENT STUDY

- The Nested PCRs (nRT-PCRs) targeting *VCA* and *EBNA1* genes were optimized with indigenously designed primers (In house primer sets) using Primer Blast Software and successfully applied for the detection of Epstein Barr virus in early stage of illness and differentially diagnosed EBV induced infectious mononucleosis from infectious mononucleosis like syndrome caused by other infective agents. It will be an excellent new addition to the armamentarium of molecular diagnostic test to detect EBV.
- PCR targeting EBNA2 and EBNA3c were standardized and applied on all clinical samples collected from pediatric post transplant patients. PCR based DNA sequencing and phylogenetic analysis on the sequences revealed predominant circulation of EBV type A genotype and also EBV genotype B in few cases. This study also revealed the occurrence of co infection by both genotypes leading to post transplant lymphoproliferative disorder.
- Real time PCR on RNA extracted from post liver transplant and pediatric infectious mononucleosis patients signified the importance of active transcription and high titer of EBV load during chronic infection leading to significant production of cytokines. Cytokines together with anti-EBV antibody raised against Epstein Barr viral antigens control EBV proliferation during acute EBV infection in infectious mononucleosis and pediatric post transplant patients and likely preventing post transplant lymphoproliferative disorder.
- Application of the standardized PCR's on ocular samples collected from patients with suspected viral retinitis revealed EBV Type A in ocular infection and also in healthy control population. This is the first time EBV genotyping based study is carried out in India. Although disease-specific genotypes or strains were not identified, this study provides useful insights into the molecular epidemiology of EBV infection in India.
- *In- vitro* study performed to determine the molecular mechanism by which EBV induces inflammation in the retinal epithelial cells revealed that EBV is capable of infecting and proliferates in adult retinal epithelial cells (ARPE-19) a process which is accompanied by the significant up regulation of viral load and both EBV type A strain and recombinant Epstein Barr viral proteins, Nuclear Antigen and p23 induced production of pro inflammatory cytokines via Toll like receptor signalling and nitric oxide leading to necrosis and cell death. These findings provide crucial information for understanding the immune mechanisms of EBV induced inflammation and cell death in ARPE and help design new immune therapeutical approaches to ocular infections caused by EBV.

10. FUTURE SCOPE OF WORK

- To identify the cell targets for EBV infection and to detect the possibility of infection in the life cycle and pathology of EBV.
- To infect Glial and astrocyte cells with EBV and to predict the immune mechanism and targets of EBV infection involved in neuronal inflammation.
- To Design small interfering RNAs (siRNA) and microRNA (miRNA) against the specific EBV induced TLR to have therapeutic application in controlling the inflammatory response associated EBV infection which can result as good therapeutic target for treatment.
- To study EBV viral transcripts and proteins and binding cites involved in infection of retinal pigment epithelial cells, to determine the crucial role of EBV in acute retinal necrosis.

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APPENDICES

APPENDIX 1: PCR AND DNA SEQUENCING:

Preparation of 0.01% Diethyl Pyrocarbonate (DEPC) coated PCR vials for RT-PCR

- To 1000ml of MilliQ water, 100µl of DEPC was added and mixed well.
- 0.5 ml and 0.2 ml new PCR vials were put in a broad beaker container and soaked in DEPC added MilliQ water and left undisturbed overnight.
- The next day water was drained and the vials autoclaved at 121°C for 15 min at 15lbs.
- After autoclaving, the vials were dried well before using for RT-PCR.

AGAROSE GEL ELECTROPHORESIS- REAGENT PREPARATION

10X TBE buffer: (pH 8)

Tris- Hcl – 54.1g

EDTA – 3.65g

Boric acid – 27.8g

Milli Q water – 500ml

Added Tris- HCl to 200ml of Milli Q water and dissolved completely. Then EDTA and boric acid was added. Mixed well and the volume was then made up to 500 ml with Milli Q water and then pH was checked. The stock 10X TBE buffer was diluted to 1X concentration before use.

GEL LOADING DYE:

- Solution A - 0.1% Bromophenol blue in 1x TBE buffer
- Solution B - 40% sucrose in 1x TBE buffer

Prepare solution A and B separately and equal volume was mixed and used for gel loading.

PREPARATION OF GEL AND VISUALIZATION OF PCR PRODUCTS:

Agarose- 0.5 g was weighed and dissolved in 25ml of 1X TBE buffer, by heating in microwave oven until no granules were seen. Then added 8µl (12.5µg) of ethidium bromide mixed well and poured to the gel tray with appropriate combs and allowed to solidify. The gel was then placed in the tank with 1X TBE buffer. 10µl amplified products were mixed with 2 - 3µl loading gel and loaded onto wells along with a molecular weight marker in each run. The electrophoresis was run at 100 Volts for 30 minutes, and the results were read and documented in the gel documentation system (Vilber Lourmat, Marne La Valle, Cedex, France).

REAGENTS FOR CYCLE SEQUENCED PRODUCTS PURIFICATION:

- **500mM EDTA:** 18.6 g of EDTA in 100 ml Milli Q water
- **125mM EDTA:**1µl of 500mM EDTA + 3 µl Milli Q water
- **3 M sodium acetate: (pH 4.6):** 2.46g of sodium acetate was first dissolved in 5 ml of water and then pH was adjusted to 4.6 with acetic acid. Then the volume was made upto 10ml with water.

APPENDIX 2: TISSUE CULTURE AND VIROLOGY:

1% Minimum Essential Medium (MEM)

- 1% Fetal Calf Serum
- Stock Antibiotic mixture-90µl/100 ml of DMEM

Preparation of Basal medium - Minimum Essential Medium (MEM):

- Add the contents of 1 unit pack of Dulbecco's Minimum Essential Medium (DMEM) to 900 ml of autoclaved Milli-Q water at room temperature with stirring until dissolved.
- Rinse the pack with a small amount of Milli-Q water to remove traces of powder and add to above solution.
- Sterilise by vacuum filtration using sterile Sartorius Millipore filter of 0.22 µm.
- Dispense the filtered medium in autoclaved medium bottles.

Preparation of Growth medium:

For preparation of growth medium, 10 ml of fetal calf serum for 90ml of basal medium, 2ml of L-Glutamine (3%), Antibiotic solutions- (Penicillin 0.1 ml, Streptomycin 0.05 ml, Gentamicin 0.05ml & Ciprofloxacin 0.05ml) and sodium bicarbonate is added until the colour changes to pale reddish orange.

Preparation of Cryo Preservation medium:

Media A:

Growth Medium (DMEM) - 8 ml

Fetal bovine serum (FBS) - 2 ml

Media B:

Dimethyl sulphoxide (DMSO) - 3ml

Maintenance medium (MEM) - 7 ml

Sterilize media B alone by filtration. Mix equal volume of media A and media B.

Maintenance of cell lines:

Trypsin EDTA solution:

TRYPSIN - 0.01 g

EDTA - 0.03g

Dextrose - 0.5g

Sterile PBS - 100ml (Filter sterilze the whole solution before use)

Subculture of continuous cell cultures:

1. Pipette out the culture medium from the tissue culture flask and rinse the cell line with trypsin-EDTA solution.
2. Add a sufficient amount of trypsin -EDTA solution (2 ml) to cover the cell layer.
3. Remove trypsin-EDTA solution.
4. Incubate the flask at room temperature/37^{0}C until cells turn oval to round in shape.
5. Cells will detach from the culture vessel surface. Tap the side of the flask with the palm of the hand to dislodge any remaining cells.
6. Add a 1 ml of growth medium and aspirate several times with a pipette to suspend and separate the cells.

7. Dilute a small sample of the cell suspension with additional growth medium and dispense directly into new growth vessels.

8. Depending on the requirement of the planned work for the day, a number of tissue culture plates or microscope well slide cultures are put up.

Preservation of cells:

1. Pipette out the culture medium from the tissue culture flask and rinse the cell line with trypsin-EDTA solution.

2. Add a sufficient amount of trypsin -EDTA solution to cover the cell layer.

3. Remove trypsin-EDTA solution.

4. Incubate the flask at room temperature /37^0C.

5. Cells will detach from the culture vessel surface. Tap the side of the flask with the palm of the hand to dislodge any remaining cells.

6. Add a 1 ml of growth medium and aspirate several times with a pipette to suspend and separate the cells.

7. Dispense equal volume of cryo medium and cell suspension into cryo vials/

8. Place cryotubes in 1°coolent.

9. Cells should be cooled from room temperature to -75^0C to -85^0C freezer.

10. The cells are then placed in the vapor phase of a liquid nitrogen freezer for long -term storage.

Recovery of frozen cells:

1. Remove cryo vial from liquid nitrogen and immediately thaw contents by placing it in a 37^0C water bath.

2. Withdraw contents with sterile Pasteur pipette, place in tissue culture flask, add fresh growth medium and incubate at 37^0C. Replace media after the cells have attached, or after 24 hours.

APPENDIX 3: LIST OF CONSUMABLES

CONSUMABLE	COMPANY
Agarose	SRL, India
Boric acid, Molecular Grade	SRL, India
Bromophenol blue	SRL, India
cDNA conversion kit	Applied biosystems
dNTPs	Bangalore genei Pvt., Ltd., India.
EDTA, Molecular grade	SRL, India
Ethanol	SD fine chemicals Pvt. Ltd., India
Ethidium Bromide	Hi Media, Mumbai, India
Molecular weight marker- 100 bp ladder	Bangalore genei Pvt., Ltd., India.
Qiamp Viral RNA mini Kit	Qiagen, Germany
Qiagen RNAse mini kit	Qiagen, Germany
Qiamp blood DNA lit	Qiagen, Germany
Taq DNA Polymerase	Bangalore genei Pvt., Ltd., India.
RPMI	Hi-media, Australia
Multi-Analyte ELISArray Kit	Qiagen, Germany
DMEM F12	Gibco, USA
QuantiTect Reverse Transcription Kit	Qiagen, Germany
oligo-dT primers	(Fermentas, USA)
DMEM F12	Gibco, USA
Primary antibodies	Santacruz biotechnology, Heidelberg, Germany
Secondary antibodies	Dako, Denmark
Recombinant viral proteins	Abcam, UK
TACS® 2 TdT-Fluor *In Situ* Apoptosis Detection Kit	Trevigen, US
FITC Annexin V Apoptosis Detection Kit	BD, US
CCK – 8 kit	Sigma, US
QuantiTect SYBR Green PCR Kit	Qiagen, Germany
Qiaquick Gel elution kit	Qiagen, Germany
RoboGene Quantification Kit	Hilden, Germany
EBV VCA ELISA kit	Demeditec Diagnostics, Germany
Griess reagent	Molecular probes, Life technologies, US
Fetal bovie serum	Hi Media, India (Source: Australia)

LIST OF PUBLICATIONS

ARTICLES PUBLISHED

1. Janani MK, Malathi J, Biswas J, Sridharan S, Madhavan HN. Genotypic Detection of Epstein Barr Virus from Clinically Suspected Viral Retinitis Patients in a Tertiary Eye Care Centre, India. *Ocul Immunol Inflamm*. 2014 Oct 17:1-8.
2. Madhuravasal Krishnan Janani, Jambulingam Malathi, Hajib N Madhavan. A Correlative Study on Epstein Barr Virus Load Determined by Real Time Pcr with Cytokine Profiles in Pediatric Post Transplant Patients. *International Journal of Scientific Research*. 2014 Aug. 3 (8): 17-20.
3. Janani MK, Malathi J, Madhavan HN. Isolation of a variant human adenovirus identified based on phylogenetic analysis during an outbreak of acute keratoconjunctivitis in Chennai. *Indian J Med Res*. 2012 Aug;136(2):260-264.

ARTICLES ACCEPTED FOR PUBLICATION:

1. Madhuravasal Krishnan Janani, Jambulingam Malathi, Andal A, Nishi Rani Singha, Hajib N Madhavan. Molecular diagnosis of Epstein Barr virus in suspected Infectious Mononucleosis pediatric patients – A pilot study in hospital based Indian population. - Journal of infection in developing countries.
2. Madhuravasal Krishnan Janani, Jambulingam Malathi, Mohamed Rela, Mohammed Farouk, Padmapriya J, Hajib N Madhavan. Genotypic detection of Epstein - Barr virus in pediatric transplant patients from India. – Indian Pediatrics.
3. Jambulingam Malathi, Madhuravasal Krishnan Janani, Nandagopal Murugan, Madhavan H Narahari Rao. Draft Genome Sequence of Epstein Barr virus strain, Isolated from a pediatric patient with post transplant lymphoproliferative disorder in India- International Journal of Pharmacy and Pharmaceutical science

ARTICLES UNDER REVIEW:

1. Madhuravasal Krishnan Janani, Shreya Kumar, H.N.Madhavan, J.Malathi . Novel Immune Regulatory Pathway Associated with Epstein Barr Virus (EBV) and the Recombinant EBV Protein Mediated Inflammation in Retinal Pigment Epithelium (ARPE-19). – Investigative Ophthalmology & Visual Science.

LIST OF PRESENTATIONS

NATIONAL-ORAL PRESENTATION

1. Presented paper entitled “Real Time PCR and PCR Based Genotyping of Epstein - Barr Virus in Pediatric Transplant Recipients” in 1st Annual conference of IAMM Tamilnadu and Puducherry chapter held on 2nd Feb 2013 at Salem.

NATIONAL POSTER PRESENTATIONS

1. Presented poster entitled " Novel Immune Regulatory Pathway Associated with Epstein Barr Virus (EBV) and the Recombinant EBV Protein Mediated Inflammation in Retinal Pigment Epithelium " in MICROCON 2014 held 17th - 19th October 2014 at Birla Auditorium, Jaipur.

2. Presented poster entitled "Standardization and application of Polymerase chain reaction targeting Viral Capsid Antigen (VCA) to differentially detect Epstein Barr Virus (EBV) from Human cytomegalovirus (HCMV) induced acute infectious mononucleosis" in CME on 28th September, 2012, at SRM, Kattankulathur.

3. Presented poster entitled “A study on an Epidemic of Acute Keratoconjunctivitis in Chennai” in IERG conference held on 30th - 31st July 2011 at Hyderabad.

4. Presented poster entitled “Standardization and application of Polymerase Chain Reaction for detection of Epstein Barr Virus in clinical samples collected from patients suspected with Infectious Mononucleosis” in Contemporary Trends in Biological and Pharmaceutical Research, March 12-13, 2011 at BITS Pilani.

AWARDS WON

1. Travel fellowship for the 19th annual meeting of Indian eye research group (IERG), Hyderabad, 2011.

2. VRF - IAMM Best paper award, IAMM (TN & Py Chapter) 2012.

3. The Young women scientist award in memory of Smt. Sreerangamma N. Narayan and Smt. Radhamani N. Anantha for the year 2014 for working in the field of Infectious diseases.

BIOGRAPHY OF THE CANDIDATE

Ms. M.K.Janani completed her under graduation in B.Sc Microbiology with gold medal from Dr. MGR university, Maduravoyal, Chennai in the year 2007. She joined off campus MS Medical Laboratory Technology conducted by BITS, Pilani and graduated in the year 2010. She worked as a Junior research fellow for 1 year and as a senior research fellow for 2 years in the project entitled "A clinico – molecular biological study on infectious mononucleosis caused by Epstein - Barr virus and to study the genotype prevalence in Chennai" funded by Indian Council of Medical research from 2010 – 2013 and working as senior research fellow for the project entitled "To Train and Develop Infrastructure for Molecular Ophthalmic Pathology and Rapid Diagnosis of Ocular Infections in an Ophthalmic Institute in Assam" from January 2014 till date. She registered for PhD in BITS, Pilani in August 2010 under the guidance Dr. J. Malathi. She has made 1 oral and 4 poster presentations in National conferences. She has 3 publications, 1 article being accepted and 3 articles are under Review. She had conducted practical and theory classes in microbiology for the under graduate (BS Optometry), Ophthalmic nursing assistant and post graduate (MSMLT) students registered under off campus courses of BITS Pilani. She have also involved in conducting Antimicrobial surveillance program conducted by WHO-WHONET 2011 at L&T Microbiology research centre and also involved in training the under graduate and post graduate students (Short term and summer training) from other institutes in various molecular biological techniques. She was awarded "Travel fellowship" to attend IERG 2011 conference, the IAMM TN-PY "Junior best paper" award for the year 2013 and the "Young women scientist award" in memory of Smt. Sreerangamma N. Narayan and Smt. Radhamani N. Anantha for the year 2014 for working in the field of Infectious diseases.

BRIEF BIOGRAPHY OF THE SUPERVISOR

Dr. J. Malathi completed her PhD from Birla Institute of Technology and Science in 2003. At present she is the Reader in the Department of Microbiology, L & T Microbiology Research Centre, Vision Research Foundation, Chennai. She has 15 years of rich experience in Medical Microbiology with 14 year postgraduate teaching in Medical microbiology with 40 publications in national and international journals. She is a recognized Ph. D guide in BITS-Pilani, and SASTRA. She had been involved in the initiation of Reverse Transcriptase PCR and Real Time PCR development of nucleic acid–based molecular biological methods in L & T Microbiology Research Centre, Vision Research Foundation, and Chennai. She is Principal Investigator of 2 projects and Co- Principal Investigator of 4 Research projects sanctioned by ICMR and DBT. Her areas of special interest are development of rapid diagnostic tests based on molecular techniques for rapid detection of infectious agents and diagnostic DNA chip for infectious diseases and to study the Molecular epidemiology of specific infectious diseases particularly related to ophthalmic infections, studying bacterial pathogenic mechanism and drug resistance. She has 40 publications in peer reviewed National and International journals. Two patents have been applied. She had done 25 presentations in National & International conferences, Invited academic lectures, National & International Symposia; CME programmes. She is a Life Member of Indian Association of Medical Microbiologists.

BRIEF BIOGRAPHY OF THE CO - SUPERVISOR

Prof. Sanjeev Kumar received his Master's degree in Biotechnology from Guru Nanak Dev University Amritsar. He received his Ph.D. degree in Molecular Biology with specialization in T-cell signaling from Institute of Medical Sciences, Banaras Hindu University, Varanasi. Later on he worked as Post doctoral fellow at Colorado State University (CSU), USA and research fellow on full time equivalent (FTE) position at National Institute of Health (NIH), Rockville, Maryland, USA. At CSU and NIH he studied the regulation of *Plasmodium* (malaria parasite) development by mosquito innate immunity. Dr. Kumar’s research is focused to understand the regulation of *Plasmodium* development by mosquito innate immune system. He is developing ways to manipulate novel mosquito immuno-active molecules which can block *Plasmodium* development and subsequent transmission among humans. He is involved in interactive teaching of first degree (M.Sc. Hons.), higher degree (M. E. Biotech) and Master of Public Health courses. The courses taught by him were General Biology, Cell Biology, Developmental Biology, Molecular Immunology, Animal Cell and Tissue Culture Technology, Laboratory, Epidemiology, Public Health and Diseases, Research Methodology. He is the Principle Investigator of 3 research projects granted by Aditya Birla Groups (ABG), Department of Science and Technology (DST) and Indian Council of Medical Research (ICMR) research respectively. He was awarded travel grants in recognition of outstanding achievements in International Congress of Entomology, National Institutes of Health (NIH), research fellowship (2003-2008), Employee special performance achievement award by National Institute of Health USA for 4 years (2004- 2008), Outstanding achievement Post-doc category poster awards, Colorado State University, Fort Collins, CO, USA (2003). He has24 publications in peer reviewed National and International journals. She had done 36 presentations in National & International conferences, Invited academic lectures, National & International Symposiums. Following are the fellowships got by him: Post-doctoral fellowship, Colorado State University (2000-2003), Post-doctoral fellowship for foreign scientists by ‘Institut National de la Santè et de la Recherche Médicale (*INSERM*)’ France (Feb-Nov 2000), Senior research fellowship, University Grants Commission (UGC), (1997-2000), Junior research fellowship, UGC (1995-1997), *Gold Medal* for outstanding achievements during M. Sc degree, Guru Nanak Dev University (GNDU), Amritsar, India (1994), Student fellowship during M. Sc. degree, Department of Biotechnology (DBT), Government of India (1992-1994).