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SEROPREVALENCE AND PHYLOGENETIC GENOTYPING OF EPSTEIN BARR VIRUS (EBV) AMONG BLOOD DONORS IN QATAR

BY

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ABSTRACT

Background: EBV is a lymphotropic herpesvirus and the causative agent of infectious mononucleosis. EBV is highly prevalent and has been linked to several malignancies. The virus is generally transferred by oral secretions and it persists as a latent infection in human B-cells. However, it can be also transmitted through blood transfusions and organ transplantations. **The goal of this study** was to (i) estimate the rate of infection of EBV in Qatar in healthy individuals, using serological testing, and viral load quantification, to (ii) study the correlation of EBV with demographic markers, and to (iii) study the molecular similarity of EBV isolates, using phylogenetic genotyping. **Materials and methods:** For estimating EBV seroprevalence, qualitative ELISA kits for detecting the following EBV antibodies were used: EBV viral capsid antigen (VCA) IgG & IgM, EBV nuclear antigen (EBNA) IgG & IgM, and early antigen (EA-D) IgG. To study the EBV viremia rate, DNA extracted from the buffy coat was subjected for viral detection and quantification using RT-PCR. For genotyping, nested PCR targeting the EBNA2 gene was used. And for further sub genotyping, the highly variable LMP-1 gene was amplified, cloned, sequenced and used for phylogenetic analysis. **Results:** Out of 673 analyzed samples (223 Qataris, 450 non-Qatari residents), 659 (97.9%) were EBV seropositive with different infection stages. Interestingly, 14 (2.1%) tested negative for all anti-EBV antibodies indicating no prior exposure to EBV. EBV DNA was detected in 354/673 (52.6%). Both EBV seroprevalence and viremia rate increased significantly with age. Genotyping for 51 randomly selected positive DNA samples showed that Genotype 1 was predominating, found in 36/51 (72.5%), while genotype 2 was found in 12 (23.5%) samples. Mixed infection was found in 2 (3.9%) samples. Surprisingly, Sub genotyping

for 30 samples revealed that all tested clones (n=119) have the South Eastern Asia 1 (SEA1) strain. 30-bp and 69 bp deletions in the LMP-1 were also found in 10% and 13.3% samples, respectively. **Conclusion**: This is the first study investigating the seroprevalence, the viremia rate, and the molecular epidemiology of EBV among blood donors in Qatar. Hence, it should provide the epidemiologists, blood banks' personnel, researchers and clinicians with EBV prevalence estimates and molecular epidemiology of EBV as a highly prevalent transfusion transmissible oncovirus.

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LIST OF ABBREVIATIONS

ALA	Alaskan
AP1	Activator Protein 1
BART	BamHI A rightward transcripts
CD	Cluster of Differentiation
CH	China
CMV	Cytomegalovirus
CR	Complement Receptor
CTAR	C-Terminal Activation Regions
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotides
EA	Early Antigen
EBER	Epstein–Barr virus Encoded small RNAs
EBNA	Epstein–Barr virus Nuclear Antigen
EBV	Epstein Barr Virus
EDTA	Ethylenediaminetetraacetic Acid
EIA	Enzyme Immunoassay
ELISA	Enzyme-linked Immunosorbent assay
GP	Glycoprotein
HBSS	Hank's Balanced Salt Solution
IC	Internal Control
IFA	Immunofluorescence Antibody Assay

IgG	Immunoglobulin class G
IgM	Immunoglobulin class M
IRB	Institutional Review Board
LMP	Latent Membrane Protein
Med	Mediterranean
MNCs	Mononuclear Cells
NC	North Carolina
NF-κB	Nuclear factor-κB
NK	Natural Killer
SOC	Super Optimal broth with Catabolic repressor
NPC	Nasopharyngeal Carcinoma
OD	Optical Density
ORF	Open Reading Frame
oriP	Origin of Plasmid Replication
PBMC	Peripheral Blood Mononuclear cells
PCR	Polymerase Chain Reaction
PTLD	Posttransplant Lymphoproliferative Disease
qRT-PCR	Real-time Polymerase Chain Reaction
RNA	Ribonucleic Acid
SEA	Southeastern Asia
TMB	Tetramethylbenzidine
TNF	Tumor Necrosis Factor
VCA	Viral Capsid Antigen

WBC White Blood Cells

WHO World Health Organization

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1- INTRODUCTION

Epstein bar virus (EBV), or human herpesvirus 4, is a lymphotropic herpesvirus and the causative agent of infectious mononucleosis. It was first discovered in cells isolated from African Burkitt's lymphoma, then it was recognized that it is highly prevalent worldwide (1). Like other herpesviruses, EBV has a latency phase, following the primary infection, it enters into the circulating B lymphocyte and persists for life in a latent state (2). It's estimated that more than 90% of world's populations is EBV-seropositive (3). Usually, the primary infection occurs in childhood, and it is asymptomatic, but when infection occurs in adults it leads to infectious mononucleosis (IM) (4). This virus has also been linked to wide range of diseases including multiple sclerosis (MS), and malignancies, such as gastric carcinoma, Hodgkin's lymphoma and nasopharyngeal carcinoma (NPC) (3-6).

EBV genome is composed of a linear, double stranded DNA with a size of 172 kilobase pairs (kbp) that encodes for more than 85 genes (4, 5). Most of the proteins encoded by the EBV genome are involved in nucleotide metabolism, to maintain the replication of the viral DNA (5, 7). In latency, only small number of viral genes are expressed, which are six EBV nuclear proteins: EBNA-1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C, EBNA-LP, and three latent membrane proteins: LMP-1, LMP-2A, LMP-2B (4).

There are two main EBV genotypes, Type 1 and Type 2, or Type A and B, respectively distinguished based on the differences in EBNA-2 gene, since the divergence in EBNA-2 reveals only 54% homology between the two types (8). EBV types 1 and 2 can further be subdivided into different virus strains (9). Most of

investigations of the genetic variability of EBV strains were based on studying the LMP-1 gene, because it showed to have a greater degree of polymorphism than most of the EBV genes. LMP-1 is a 356-amino acid protein that plays an important role in signal transduction and cell survival. Variants in LMP-1 were classified into 7 main groups: B95-8, Alaskan, China 1, China 2, Med+, Med- and NC (3, 5, 10). However, new strains were reported from different origins. In Thailand, two other new variants were found which were named : Southeastern Asia 1 (SEA1), and Southeastern Asia 2 (SEA2). CAO variant was also isolated from nasopharyngeal carcinoma patients (11, 12) Interestingly, it was reported that multiple EBV variants can be detected within one individual. These variants were also correlated with cancer progression. A variant with 30 bp deletion, was isolated from NPC tumor, and showed to have a higher transforming activity than the typical LMP-1 (13).

EBV is transmitted through the oral rout, which is the primary rout of transmission; however, it has been reported that transfusion and organ transplantation are also routes of EBV spread (14-16). Although there are efforts from blood banking organizations to minimize the risk of transfusion transmission, through screening for numerous infectious pathogens, there it is still a concern regarding transmission of untested pathogens, such as EBV (17). Blood banks rely on the leukoreduction procedure to ensure the safety of blood products, but although leukoreduction procedure can pointedly reduce the number of EBV genome, it was found that EBV can be still detected in leukoreduced products. In conclusion, leukoreduction doesn't completely eliminate the EBV carrying cells, and this signals a potential risk for blood products recipients, especially if they are immunocompromised (17, 18).

Several studies were conducted to investigate the presence of EBV genome in healthy blood donors. Many studies also have shown the geographic distribution of EBV strain variants among healthy infected carriers. However, to the best of our knowledge, no study have been conducted in Qatar concerning EBV detection and genotyping in neither cancer patients nor healthy donors. Accordingly, **the aim** of this study was to answer preliminary questions about EBV in Qatar; through studying the rate of infection of EBV in the country, the demographic distribution of the virus in relation to gender, age, and ethnicity in Qatar, and how genetically similar tested strains are to previously reported EBV strains. This work aims to help the blood banks in Qatar by providing them with preliminary data about EBV viremia, as a blood transmitted oncovirus, hence, improving the health standard of the community by providing a better standard of health services.

Hypothesis

Qatar is Multinational country, with a mixed population. We hypothesize that: Multiple or new EBV variants are expected to be found in healthy blood donors in Qatar.

2- LITERATURE REVIEW

2.1 EBV History and Discovery

The discovery of EBV started with the identification of a novel lymphoma affecting young children in central Africa, which was first reported by Denis Burkitt in 1958 (19-21). The unique geographical pattern of the disease distribution, which was related to environmental conditions, where equatorial rainy areas were more affected, drew the attention towards the hypothesis that an infectious agent could be involved in the transmission process (22, 23). In 1964, Epstein-Barr virus was first identified by Epstein, Achong, and Barr in cultured Burkitt's lymphoma cells. Using electron micrographs, they showed a herpesvirus like particles with unique antibody reactivity, not similar to any other known herpesviruses. Then, in 1965, it was confirmed that a new human virus was officially identified which belongs to the herpes family, and named Epstein-Barr virus (23).

2.2 EBV Structure and Genome

The EBV virion structure is similar to other herpesviruses. It consists of a toroid shaped protein core wrapped with the viral DNA inside an icosahedral capsid of 162 capsomers, a viral tegument containing a protein that lines the space between the nucleocapsid and the outer envelope. Different glycoprotein spikes are inserted into the viral envelop (5, 24).

EBV genome is composed of a linear, double stranded DNA with a relatively large genome size of ~ 172 kilobase pairs (kbp) that encodes for more than 85 genes (4, 5). In order to have the highest coding capacity, the viral genome is divided into short and

long unique sequence domains by a series of around 540 bp terminal direct repeats and around 3.1 kbp large internal repeats (7, 25). These repetitions serve as an indicator to determine whether the source of EBV in the infected cells came from the same progenitor cell. That is, when EBV infects a cell, the viral DNA persists as a circular episome, that when passes to the future generations, it retains the same number of terminal repeats during the latent phase of infection (5). The nomenclature of the EBV open reading frames (ORFs) was established according to the BamHI-restriction fragments, where they were found and ordered in descending order from A to Z, based on the fragment sizes. The fragments were also divided into latent or lyric genes (5, 24).

Most of the proteins encoded by EBV genome are involved in nucleotide metabolism (to maintain the replication of the viral DNA) and to build the structural compartments of the virus such as nucleocapsid, tegument proteins, and the envelope (7). Additionally, EBV genome consists of several latent genes that are non-translated during the lytic phase and number of latency associated RNA genes that are expressed during latency (5, 7). During latent EBV infection, the viral genome persists for life-long in multiple circular episomes inside the infected cell nucleus. To maintain this episome like plasmids during cell division, two components are needed: a cis-acting DNA segment (oriP), and a trans-acting nuclear protein (26). In latency, only few viral genes are expressed, which includes the six EBV nuclear proteins: EBNA-1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C, EBNA-LP, plus three latent membrane proteins: LMP-1, LMP-2A, LMP-2B (4). Furthermore, although EBV DNA usually persists in the form of episome, it was found that EBV DNA can integrate into the cell chromosomal DNA, and persist as integrated DNA as well (27).

2.3 EBV Types and Strain Variation

It has long been known that there are two different EBV genotypes: Type 1 and Type 2, also known as Type A and B, respectively (25). These two types were distinguished based on the differences in EBNA-2 gene, since EBNA-2 clearly classifies Type 1 and Type 2, where the rest of the EBV genes differ only by less than 5% in their sequence (3, 5). EBNA-3 gene family show variations between EBV types, but with less degree of sequence difference than EBNA-2 gene (3). The divergence in EBNA-2 reveals only 54% homology between the two types, facilitating the distinction between each EBV type (8). Interestingly, it was found that EBV types noticeably differ in their transformation abilities, for instance, Type 1 transforms B lymphocytes more willingly than Type 2 *in vitro,* and when a recombinant Type 2 virus acquired Type 1 EBNA-2A gene, it gained the transforming ability of Type 1 virus (28). In this study, the EBV were typed by nested PCR using different sets of primers that either recognize type 1 or 2.

EBV types 1 and 2 can further be subtyped into different EBV strains (9). The genetic variability between EBV strain is thought to occur due to the nature of the EBV life cycle within the lymphocytes. For instance, when the EBV infected lymphocyte passes through the germinal center of the lymph node, which is considered as a highly mutagenic environment, increased rate of mutations could occur, , and thus, EBV also can induce errors during replication, creating more genetic variability between individuals (29). There are many studies in the literature that have focused on investigating the genetic variability of EBV strains and tried to correlate this variability to the geographic areas and to the disease outcomes. In these studies, genes which were

identified to have an important role in EBV viral life cycle were sequenced such as BZLF1, EBNA-1, EBNA-2, EBNA-3A, -3B, and -3C, LMP-1, and LMP-2a (4).

Among the aforementioned genes, most of the recent investigations on EBV strain variation were based on studying the LMP-1 gene sequence, because it has showed to have a greater degree of polymorphism than most of EBV genes between different strains (3). LMP-1 is a 356-amino acid protein, which consists of a short cytoplasmic N-terminus, six membrane spanning domains, and a long cytoplasmic C- terminal domain (30). The C-terminal domains (CTAR1, 2, and 3) of LMP-1 play an important role in signal transduction through mimicking the CD40-mediated signaling, and as a result, they work as homologous to the TNF-receptor family in the B lymphocytes and epithelial cells (5). Moreover, they regulate the NF-κB and cell survival in various pathways. Therefore when mutated, sequence variation can affect cell process directly as it interferes with major signaling pathways (3). It is well known now, that LMP-1 is essential in the transformation of B lymphocytes into lymphoblastoid cell line, and it has the ability to block apoptosis as a result of upregulating different anti-apoptotic proteins such as A20 and Bcl-2, and inhibiting the p53-mediated apoptosis (5).

Based on the LMP-1 sequence variation, EBV strains were classified into 7 main groups (variants) relative to the wild type strain B95-8. Naming of these variants reflects their geographic origin or from where they were driven: Alaskan (Ala), China (Ch1) and (Ch2), Mediterranean (Med+) and (Med-) and North Carolina (NC) (3, 10). However, new strains were reported from different origins. In Thailand, two other new variants were found which were named : Southeastern Asia 1 (SEA1), and Southeastern Asia 2 (SEA2). CAO variant was also isolated from nasopharyngeal carcinoma patients (11, 12)

Multiple EBV variants can be detected within one individual, as a patient can be infected with more than one type (9). There are evidences of specific multiple LMP-1 variants found in people infected with infectious mononucleosis, as well as in other EBV associated malignancies such as Hodgkin Lymphoma and Nasopharyngeal carcinoma (NPC). In HIV patients, multiple EBV strains can be present (3). Interest in LMP-1 variants was increased, when findings correlated LMP-1 variants with specific cancers. For example, a variant with 30 bp deletion was detected frequently in NPC patients and this variant showed higher transforming activity than the typical LMP-1 (13). Furthermore, a 69 bp deletion variant has also been reported in Burkitt's lymphoma and in a lesser rate with NPC. Further, the 69 deletion was also correlated with decreased cell AP1 transcription factor activation (3, 31). Several reports also investigated the presence of LMP-1 variants among healthy carriers (9, 32, 33). A recent study compared the prevalence of EBV genotypes and del-LMP-1 among Polish, Taiwanese and Arabic healthy individuals revealed that 62.5% Taiwanese and 55.6% Polish had 30-bp deletion in the LMP-1 gene, however, Arabs didn't have this deletion (33). Another study investigated the frequency of 30 bp deletion in EBV healthy carriers from Argentina and found that it is present in 28% of these healthy people (32) . However, it is still not known how many EBV variants can be found in one individual, and whether the immune system of a previously infected individual provides protection against new multiple variants (29).

2.4 EBV Viral Tropism (activation)

Many studies have been conducted to investigate the EBV host cell interactions, and the latency associated with EBV infection in different cell types and various medical conditions. These studies have investigated virus cell interaction in different infection conditions, such as in malignancies, lymphoproliferative disease in transplantation and in the normal B lymphocytes during infectious mononucleosis (34). In normal hosts, B lymphocytes and epithelial cells are the cellular targets for EBV primary infection. However, EBV can infect wide range of non-B lymphocytes and it critically affects the development and pathogenesis of EBV related diseases (35). Early studies reported the presence and replication of EBV viral particles in the oropharyngeal epithelial cells of patients with acute infectious mononucleosis (36, 37), and in epithelial cells of HIV patients suffering from oral hairy leukoplakia (38). More recent studies showed that the tonsil epithelium of asymptomatic patients has the ability to carry EBV infection, which is a part of the viral life cycle (39). Furthermore, EBV has the ability to infect T lymphocytes, plasma cells, NK cells, monocytes, follicular dendritic cells squamous, myoepithelial and glandular epithelial cells, and also smooth muscle cells (40-44).

Despite the wide range of suspected cell types that can be involved in EBV infection, it is believed that B lymphocytes have a critical role in the viral life cycle, as agammaglobulinemia patients, who have a genetic mutations that leads to the absence of mature B lymphocytes, are not affected with EBV (45). Primary B lymphocytes can be easily infected with EBV, because B lymphocytes possess a major receptor molecule of the virus called cellular complement receptor type 2 (CR2 or CD21), which binds to EBV glycoprotein gp350/220 (46). On the other hand, the interaction of EBV with epithelial

cells is less understood. It was believed that epithelial cells acquire the infection through transfer from EBV-coated B lymphocytes (34). *In vitro* studies showed that a low rate of infection was achieved when epithelial cells were exposed to cell free virus preparations, while a quantifiable level of infection was reached when epithelial cells were cultured with EBV infected B lymphocytes. This prompts the idea of the importance of B lymphocytes in the initiation of infection, as EBV must go through an initial cycle of replication in a primary B lymphocyte before it reaches epithelial cells (47). EBV also might enter the epithelium through the surface of resting B lymphocyte. B Lymphocyte can act as a transfer vehicle, to transfer EBV infection to CD21 negative epithelial cells after EBV binds to its surface (47). However, it is still in doubt whether B lymphocytes or epithelial cells. are the primary target of EBV spread (7).

2.5 EBV Viral Life Cycle

Usually EBV spreads through saliva, and upon entry of the virus, it enters the epithelium of the tonsils, where it starts the lytic phase of infection that involves virus replication (5). Infected naive B lymphocytes become activated lymphoblasts and migrate to the lymph node follicle to initiate a reaction in the germinal center of the follicle using the "latency III" program, where all latent growth proteins are expressed and adversely regulate EBV growth. This includes EBV nuclear antigens (EBNA-1, -2, -3, -3A, -3B, -3C, and -LP), and latent membrane proteins LMPs (LMP-1, -2A, and -2B) (5, 46). Type II latency program then is initiated in which only EBNA1, the EBERs, the BARTs, LMP-1 and LMP-2A are expressed (46) and survival signals will be provided to cells to move out of the germinal center as memory B lymphocytes (5). "Latency 0" begins in the

memory B lymphocytes, and it is characterized by turning off all viral proteins expression (5). If EBNA-1 gene only is expressed when these memory B lymphocytes divide, then this is called “latency type I” (26, 48). Infected memory B lymphocytes can also migrate back to the tonsils, where they can elicit more viral replication that can spread and infect other B lymphocytes as well (2). In the primary infection, T lymphocytes are responsible for eliminating the newly infected cells and to control the infection, however, during latency, EBV is hidden from the immune system as it remains silent in the resting memory B lymphocytes with no expression of any viral protein (5, 49).

Viral reactivation can happen occasionally in latently infected memory B lymphocytes and leads to a new viral cycle, where it replicates, infects new cells, and sheds in the saliva (46). Under healthy conditions, immunocompetent individuals can have EBV reactivation with no specific symptoms due to the infection control by the cytotoxic T lymphocytes (50). However, EBV reactivation can be life threatening in patients under immunosuppression and can lead to severe EBV related pathologies such as post-transplant lymphoproliferative disorders (PTLDs) (50). There are several described causes of EBV reactivation, such as the presence of foreign antigen that leads to memory B lymphocytes division, which in turn can induce viral reactivation and replication (51). This means that any new infection can lead to EBV reactivation (5). For instance, malaria infection have been linked to EBV reactivation, as *P. falciparum* antigens can directly trigger EBV reactivation, and therefore can increase the risk of developing Burkitt’s lymphoma in malaria endemic areas (52). The cystein-rich inter-domain region 1α (CIDR1α) in the *P. falciparum* membrane protein 1, was found to activate the memory B lymphocytes where EBV resides (52). Another cause is

immunodeficiency and immunosuppression, due to altered immune system. In this case, uncontrolled reactivation of EBV may occur and can lead to various lymphoproliferative diseases (50). Other factors, such as inflammation and chemical agents or drugs, have been also linked to EBV reactivation from latently infected cells (5).

2.6 EBV Prevalence and Transmission

In healthy individuals EBV is highly prevalent, as it affects more than 90% of individuals around the world (53). The age of primary infection was found to vary according to socioeconomic factors that are reflected by crowdedness and low sanitation (5). EBV seroconversion occurrence has two patterns. In developed counties with high hygiene standards, EBV seroconversion peaks in children between 2 to 4 years and also in 14 to 18 years, and it increases with age, ranging from 0 to 70% at childhood and reaching to more 90% in adulthood (17). In contrast, countries with less hygiene standards, EBV is usually acquired in early childhood, and almost all children in those developing countries are seropositive by the age of 6 years (54).

EBV types occur worldwide but they differ in their geographic distribution. EBV type 1 is prevalent in Europe, America, China, and South Asia, while EBV type 2 is less prevalent in these populations and it is more observed in African and Papua New Guinean populations, where it shares an equal distribution with type 1 (5, 55). Immunocompromised patients are more susceptible to acquire both types (5). However, heathy individuals (with less frequency) as well can have mixed infection with both type 1 and 2 (9). In my study, I found that EBV type 1 is more prevalent than type 2, yet, mixed infection was also detected.

The main route of EBV transmission is the oral route, as it is generally transmitted through saliva that contains infected epithelial cells (54). Also, it can spread through blood, blood transfusion and organ transplantations (14, 16, 17, 56). Infected epithelial cells can also be found in the uterine cervix or in the semen, suggesting the possibility of EBV spread through sexual contact (54). Kissing, sharing personal objectives such as toothbrushes, eating utensils, or sharing food and drinks with an infected individual can all lead to EBV spread (56).

2.7 EBV in Blood Transfusion and Organ Transplantation

It has long been known that blood transfusions and organ transplantations can be routes for EBV transmission, as reported in 1969 by Gerber et al. (14). In this study, the authors showed that patients receiving donor blood during an open heart operation acquired EBV, indicating the possibility of EBV transmission by blood transfusion. Furthermore, early studies revealed that EBV transmission can be through organ transplantation, where patients developed infectious mononucleosis after transplantation (15, 16). Reports showed that a healthy EBV seropositive individual carry around 0.1 to 50 EBV infected B lymphocytes per 1,000,000 peripheral blood mononuclear cells (MNCs). Therefore it's possible that EBV can be transmitted through white blood cells of the blood (17, 57).

Multiple studied were conducted to estimate the prevalence of EBV DNA in the blood of healthy blood donors., Results revealed that EBV DNA detection in blood donors varies from low to high rates. In US, a study showed that 72% of a 100 randomly selected blood donors had a detectable EBV DNA, suggesting that the potential for

transfusion-mediated transmission EBV is high (58). In Japan, randomly selected blood donors were tested for the presence of EBV DNA and results showed that EBV DNA was detected in 39.5% of the donors (59). Another recent study in Burkina Faso showed a lower level of EBV rate among blood donors, as it was detected in only 5.1% of the donors (60). In this study, 52.6% of blood donors have detectable EBV DNA in their peripheral blood.

EBV has been linked to the development of post-transplant lymphoproliferative disorders (PTLD's), which are a group of heterogeneous diseases that develop in immunocompromised patients after receiving a solid organ or hematopoietic stem cell transplant (61). The incidence of lymphoproliferative disorders increases with solid organ and bone marrow transplantations (62, 63). These disorders develope as a result of immunosuppression, and they vary from benign slow polyclonal proliferations to overtly malignant monoclonal proliferations of lymphocytes and plasma cells (61, 64). PTLD was first reported in 1968 in two renal transplantation recipients, and it was linked to the immunosuppressive therapy that was administered to the patients (65). Mortality from PTLDs is high with no progress in the outcomes over the years. In fact, the highest risk of developing PTLDs was in the first year after initiation of transplantations (66). The World Health Organization (WHO) classifies PTLDs to: (i) early lesions of polyclonal or oligoclonal lymphoid proliferations that are mainly derived from EBV infection; and (ii) late monoclonal lymphoproliferative diseases that are not necessarily associated with EBV, including polymorphic and monomorphic PTLDs, which also can be subdivided into Burkitt's lymphoma, Burkitt's-like lymphoma, diffuse large B-cell lymphoma and Classical Hodgkin Lymphoma (67).

It has been known that oncogenic herpesviruses like EBV and HHV-8 are involved in the development and pathogenesis of PTLDs, because these viruses have the ability to directly infect and transform B lymphocytes (67). Indeed, EBV was found to be present in up to 2/3 of PTLD cases (61). A higher risk to develop PLTD is found in EBV negative than in EBV positive recipients regardless the status of the donor, but the highest risk is when the recipient is EBV negative, and the donor is EBV positive (68).

In EBV infection, when the viral genome presents in an episomal form in the latent period of infection, few latent genes are expressed in different latency programs and these genes code for proteins that are found to have a direct oncogenic effect (5). When immunosuppression is severe, EBV positive cells express latency type III program, and transformed B lymphocytes grow continuously in the absence of effective EBV specific cytotoxic T cells (2). Genes that are expressed include EBNA-3 group (EBNA-3A, -3B, and -3C), which are extremely oncogenic (5). Increased EBV viral load, and decreased cytotoxic T cells is strongly linked to the development of PTLD (61). When autologous EBV specific cytotoxic T cells were used to treat PTLDs, EBV viral load was controlled and tumor size was reduced (67), which confirms the strong association of EBV to this disease.

The efforts to prevent transmission of EBV from EBV positive donors is based on the process of leukoreduction, which was introduced in 1999, and aims to remove the white blood cells from various blood products (69). Qu et al. concluded in a study of leukoreduction efficacy, that EBV PCR negative blood products after leukoreduction, are expected to have a very low probability of transmissible EBV, and the risk is highly reduced (70). However, in another recent published in the international journal of

transfusion medicine, a group of scientist found that EBV was detected in one platelets bag after leukoreduction (18). This data shows a clear evidence that leukoreduction doesn't rule out the possibility of EBV transmission, and leukoreduced blood products can harbor EBV. Consequently, there is a potential risk in immunocompromised patients who are more vulnerable to EBV infection, and patients who receive large volumes of blood (18).

Although the process of leukoreduction decreases the viral copies present in the WBCs, it is important to draw the attention that the risk of EBV transmission, or other certain blood transfusion viruses, is not completely eliminated, and transmission is not 100% prevented by leukoreduction (17). For instance, a study has shown that CMV seronegative blood units can enhance the safety of blood transfusion more than leukoreduced blood products in at risk patients like organ transplantation and immunosuppressed patients. Thus, leukoreduced blood products are not equal to seronegative blood products with regard to safety, and this might be applied for EBV as well (17, 71).

2.8 Detection of EBV

The clinical presentation of EBV infection usually overlap with other acute viral syndromes caused by other viruses such as hepatitis viruses and cytomegalovirus (CMV), which can lead to similar symptoms (72). This emphasizes the importance of having reliable laboratory diagnostic tools that help in the differential diagnosis. Diagnostic schemes of EBV differ according to the patient's immune condition, because the importance and urgency of therapeutic intervention differ between immunocompromised

and immunocompetent individuals. Testing for anti-EBV antibodies was and still the most widely used laboratory assay to evaluate the infection. This includes nonspecific tests such as hetetophile antibodies detection (mono spot test), and a wide range of EBV specific serological assays such as: ELISA, EIA, IFA, chemoluminescence, immunoblot, and IgG avidity. In addition to the serological testing, molecular assays for nuclear acid detection such as in PCR, *in situ* hybridization and in situ PCR are also important (73). Other diagnostic tools also have been used in detection of EBV-associated tumors such as immunhistochemistry and immunocytology (73, 74).

2.8.1 EBV Serological Testing:

Serological testing is based on the detection of EBV antibodies in the patient's serum. Although the serology for EBV diagnosis shows a high degree of variability, it's still preferred and commonly used, as it provides a reasonable criteria to identify the patient's infection status (73). EBV genome codes for different structural and nonstructural genes, some of these genes are used in the serological diagnosis, as the humoral response produces antibodies against the product of these genes. This includes: the gene that codes for the viral capsid antigens (VCAs), the early antigens (EAs), as well as the genes that code for Epstein Barr nuclear antigens (EBNAs) (72, 73). Heterophile test is also one of the commonly used tests to help in the clinical diagnosis. This test is based on detecting heterophile antibodies which are antibodies that agglutinate erythrocytes from animal source, and are mainly linked to mononucleosis caused by EBV infection or infrequently by other diseases (75). This test is easy to perform, inexpensive and commercially available, but it lacks specificity, as false positive results were reported

in other non EBV conditions such as in autoimmune diseases and cancer (76). Moreover, this assay shows low sensitivity with high false negative results when used for children younger than two years old, as they might lack specific EBV antibodies (75, 77). In immunocompetent individuals, usually at least three serological parameters are needed to detect EBV antibodies: VCA-IgG, VCA- IgM, and EBNA-1 IgG (78). Detection of IgG antibodies to EBV early antigen EA can also be done and helps in differentiation of the EBV diseases status (73, 75) (Figure 1)

Viral capsid antigen (VCA) is a complex of 7 structural proteins and glycoproteins, and it is synthesized in the lytic cycle of EBV replication (79). Serodiagnosis of VCA is based on the detection of antibodies against the two recombinant viral capsid antigens: the N- terminal of full length p23 and the carboxy half of p18 (80). These two proteins were joined *in vitro* by autologous gene fusion in 1999, which then established the base for developing novel EBV ELISAs (80, 81). Detection of VCA antibodies involves the two immunoglobulin classes IgG and IgM. The humoral response to the VCA complex is typically found early at the onset of clinical symptoms (76) . In a study investigating EBV status in college students, VCA-IgM was detected by enzyme immunoassay 8 days earlier than the onset of symptoms (76). VCA- IgM is produced transitionally and used as an indicative of recent primary infection. That is, VCA-IgM is no more detected after convalescence, and generally it doesn't occur another time in life (73). Although VCA IgM appears early and helps in the diagnosis of acute EBV infection, there are some limitations that interfere with the accurate interpretation of the results. For example, some children and adults might have negative VCA-IgM in primary acute infection (75), and EBV-IgM cross-reactivity with other antigenically

related infections especially CMV infection was also reported (82, 83). VCA- IgG is found in acute, convalescence or past infections, as it starts to appear at the same time as VCA-IgM (73, 75, 80). Antibodies against the p18 components develop after p23 antibodies, and then persists for life as an indication of EBV exposure (76). Measuring VCA- IgG antibodies is found to be best single test to indicate a previous EBV infection as all patients with infectious mononucleosis produce IgG antibodies to VCA (84).

EBV nuclear antigen (EBNA) is composed of 6 proteins (EBNA-1, -3, -3A, 3B, 3C and LP) (2) . EBNA-1 is expressed in all EBV infected cells, and IgG against this protein is a late marker of EBV infection (84). EBNA-1 IgG antibodies appear late, 3 to 6 months after the time of disease, then they decline but continue to present in a detectable level for life Thus, detection of EBNA-1 antibodies indicates past or recovering EBV infection (5, 84). However, VCA- IgG indicates past infection more accurately than EBNA- IgG, because EBNA- IgG is never developed in around 5%- 10% of EBV infected healthy individuals, and this percentage is higher in immunocompromised patients (76, 85). Usually antibodies to EBNA are tested by standard immunofluorescent assays and enzyme immunoassays. However, EBNA enzyme immunoassays may give false positive results (56, 75, 78). The IgM class of EBNA-1 in not usually measured, but when detected, it indicates a recent primary infection, however, it may persists for several months after the primary infection and it can reappear again in reactivation (75). EBNA-1 IgM has cross reactivity with other viruses such as CMV and Parvovirus B19, and it may show false negative results (86, 87).

Early antigen (EA), is a complex of nonstructural proteins that are expressed by EBV infected cells in the lytic phase. It is composed of two components: diffused EA-D and restricted EA-R (88) . IgG antibodies against EA are detected transiently in up to 3 months or more during infection mononucleosis (85). Usually the humoral response is against the D component, however, children undergoing silent EBV seroconversion might also produce antibodies to the R component (75, 89). High levels of EA-R antibodies have been detected in Burkitt's lymphoma (75), and can also be indicative of reactivation of a latent EBV infection (90). In contrast, high titers of EA-D were found to be produced in nasopharyngeal carcinoma patients (91). Hence, detection of only EA antibodies can't serve as a definitive diagnosis to identify the EBV condition, because high titers are found in different diseases, and in healthy individuals as well (92). Usually, EA antibodies appear in the acute phase and then declines to undetectable levels, but studies showed that only 60% to 85% of acute infection patients have EA positive results (73, 76) and 20% to 30% of healthy individuals with past EBV infection have detectable levels of EA antibodies (73). Because of these reasons, the diagnostic value of these antibodies is still debatable (75). Nevertheless, combining EA antibodies testing with other diagnostic tools can be useful in the diagnosis.

In general, EBV infection in immunocompetent patients is detected and classified using the previously mentioned antibodies in patients' sera, but when results are uninterpretable or can't clearly distinguish the stage of infection, other assays can be done to confirm the suspected diagnosis, such as western blot, immunoblot, and more commonly, the IgG avidity testing (76, 93).

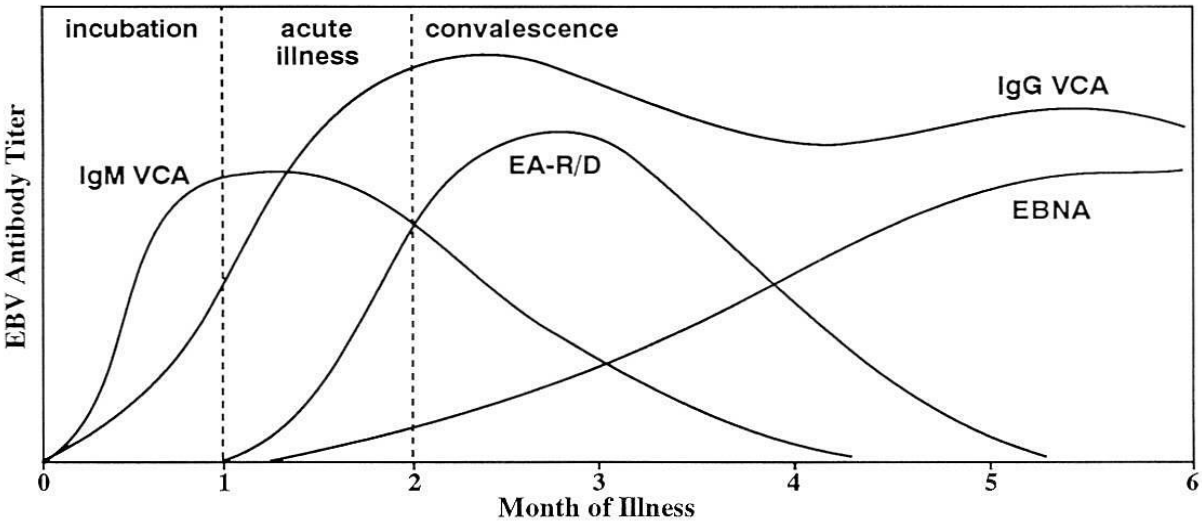

Figure 1. A scheme of serological response to EBV infection. VCA IgM is detected in the active phase of infection, and then declines in convalescence. VCA IgG increases at the same time of VCA IgM but it remains positive for life indicating past infection. EBNA antibodies are detectable late in the phase of infection and also remain positive. EA antibodies to the class R or D increase in the acute phase on infection and decline after convalescence. Adapted from Johnson DH, 1995.

IgG Avidity Assay:

Due to the high variability and cross reactivity in EBV serological responses, mainly with the VCA antibodies, more parameters are occasionally needed for the confirmation of infection condition. IgG avidity assay is usually used in combination with other serologic markers. It is based on the principle that during the acute phase of infection, the binding strength of EBV IgG antibodies to their target antigens is not as high as the antibodies binding strength after finishing the acute infection, as the antibodies undergo maturation (75, 94). Treating low avidity IgG antibodies with urea or chaotropic reagent leads to antibodies disassociation. Consequently, the difference in the antibodies amount before and after urea treatment is evaluated to determine the avidity strength which in turn represents the stage of infection and distinguish acute from past infection (76, 95). This method was found to be a reliable tool in EBV primary infection confirmation in patients with undetectable VCA-IgM, and in the differential diagnosis as well (93, 95).

2.8.2 Molecular Assays

Various molecular techniques have been developed and applied to detect EBV DNA and to quantify the viral load (40, 96-98). In situ hybridization, RNA and protein based assays, detection of EBV DNA in blood samples by quantitative real time PCR (qRT-PCR), Southern blotting and Dot blotting have all been used in the diagnosis and monitoring of primary EBV infection, reactivation, and in EBV related diseases (40, 99). These methods aid in the diagnosis, but due to the lack of standardization, the difference

in sensitivity and specificity from laboratory to laboratory should be always considered (75).

There are many studies that have shown the importance of using qRT-PCR as a sensitive and reliable method and a complementary tool to other serologic markers, in particular, for diagnosis of EBV primary (acute) infection and EBV silent reactivation (50, 77, 100, 101). More importantly, this method is very crucial and widely used in monitoring the immune status for immunocompromised patients and in patients at risk of developing EBV-related diseases (102-104). However, the threshold value in which medical intervention is required, the units of measurement, and the best specimen to be used for DNA testing are still questionable and not standardized (75). Moreover, there is still no consensus on the ideal method for performing qRT-PCR for EBV detection and quantification, and this increases the variability between laboratories and between studies (40). Different detection methods are available commercially, some commercial primers and probes target include: LMP-2 gene, BHRF-1 (a transmembrane protein), BKRF1 (EBNA-1 gene), BNRF1 (a major tegument protein), BXLF1 (thymidine kinase), BZLF1 (ZEBRA) or BALF5 (viral DNA polymerase) (40, 75). The unit of measurement also varies; it can be reported as copies per DNA concentration as copies per microgram of DNA, or copies per milliliter, copies per 100,000 white blood cells, and copies per positive cell (40, 75, 105). Samples that were used qRT-PCR assays are various, including: serum, whole blood, tissue biopsy, and peripheral blood mononuclear cells (PMNCs). Although there is still much debate concerning these issues, but in general, the best specimen to use is based on the patient's condition and the stage of the disease. Studies of EBV life cycle have showed that the production of EBV virions during the

acute phase of infection and the degradation of EBV DNA by apoptotic cells, both lead to the spread of virions and free or degraded EBV in the peripheral blood of the patient. Therefore, this allows for EBV DNA detection in patients' peripheral blood (99, 106, 107). In latent phase of infection, transformed B lymphocytes also travel to the blood (75). Consequently, EBV DNA in acute infection can be detected in the serum or in the unfractionated blood, as well as in the PMNCs.

EBV DNA in acutely infected patients can be detected within two weeks of the onset of symptoms and it reaches its peak during this time (75). Then, after the initiation of immune response, the viral load starts to decrease rapidly to low or even undetectable levels in the plasma or serum (100), but decreases slowly in the cellular portion of blood, where it remains latent in the memory cells for long time, thus it can be detected if the sample was PMNCs (40, 108). However, it is important to consider the individual differences in EBV kinetics between patients as the viral load might take up to one year to reach a stable low level in some individuals according to immune status and the patient's condition (40, 109). Studies showed that healthy individuals carry a stable number of EBV infected cells (82). In a healthy carrier, latently infected memory B lymphocytes harbor EBV genome, approximately, per 1 million leukocyte, there are 1-50 copies of EBV DNA, while in serum or plasma EBV DNA is almost below the limit of detection (40) for the same individual. Therefore, the ability to detect EBV DNA in serum might serve as useful indicator for EBV primary infection or reactivation. Patients with active infection or with EBV related cancers have been found to have higher viral load in their cell free blood (40).

EBV DNA detection in patient's serum can be useful especially in the early stages of the acute infection, and it was reported that it is more sensitive than serology and IgG avidity testing (100). However, it's not necessary to be performed for immunocompetent patients, as serology is sufficient unless a result was indeterminate and an additional test was needed (110), or when EBV infection is strongly suspected with negative serology results (111). In EBV-associated diseases, the sample of choice differs based on the type of disease, for example, serum can be useful in detecting EBV DNA in Hodgkin's lymphoma patients as the biology of the disease includes migration of episomal or apoptotic cells derived EBV DNA to the bloodstream, therefore , plasma or serum samples are desirable for EBV quantification (99). Similarly, in nasopharyngeal carcinoma, cancer cells proliferate in the tissue and uncommonly migrate to the peripheral blood, but cell free EBV DNA can be detected in the peripheral blood using a serum or plasma sample (99). In contrast, in PTLD, the disease biology involves blast B lymphocytes migration to the bloodstream, accordingly, using a PMNC specimen in preferable (99). Furthermore, it was found that the viral load correlates with the severity of the disease in EBV associated malignancies and lymphoproliferative diseases and can be used as a prognostic marker (108, 112).

In conclusion, qRT-PCR is an important diagnostic tool especially in immunocompromised patients, where serology results may be confusing and unclear due to the incomplete humoral response. Furthermore, measuring the viral load by qRT-PCR is considered the best test to diagnose, monitor and anticipate the risk of developing EBV related disease in these patients (76, 113).

3- METHODOLOGY

3.1 Sample Collection and Ethical Compliance

Whole blood samples were collected in EDTA tubes from healthy donors at the Blood Donor Unit at Hamad Medical Corporation over a period of one year (Sep 2014 – Sep 2015). Blood samples were handled and stored following standard safety procedures and guideline. This study was approved by the Institutional Review Board (IRB) of Qatar University: (QU-IRB 518-EA/15). The study doesn't include any harm to the participants. Participants' rights were not harmed, because blood samples obtained from the blood bank were anonymous. The only information obtained were those related to nationality, sex, and age. All participants' data was kept confidential.

3.2 Sample Size

Upon literature review, no specific information or estimates on EBV viremia rates or genotypes in Qatar were found. As this is the first study of its kind to be conducted in Qatar and, hence, due to non-availability of precise information related to primary outcome no formal sample size calculation is feasible for the present study. The total number of blood samples that were collected were 673 , 223 (33%) from Qatari donors and 450 (67%) from donors of other nationalities.

3.3 Ani-EBV Antibodies Testing

3.3.1 Qualitative ELISA Testing of Anti- EBV Antibodies

For the seroprevalence study, ELISA kits (Diagnostic Automation, USA) were used to screen plasma samples for the presence of EBV antibodies. A panel test was performed; this included screening antibodies for: viral capsid antigen (VCA) [both VCA- IgG (Catalog # 1405-2), and VCA- IgM (Catalog # 140692)], Epstein Barr nuclear antigen -1 (EBNA-1), [both EBNA-IgG (Catalog # 1425-1), and EBNA-IgM (Catalog # 1426-1)], and EBV early antigen (EA)-IgG (Catalog # 1415-11).

All ELISA assays were based on the same principle. In each kit, purified EBV antigens were coated on the surface of polystyrene microwells to bind to the specific complementary antibodies, if present in the tested sample, forming antigen-antibody complexes. Excess antibodies were then removed by washing. After washing, anti-human IgG conjugated with horseradish peroxidase was added, which will bind to the antibody-antigen complexes. A second washing was then performed to remove excess conjugate, followed by adding of a substrate (Chromogen solution, tetramethylbenzidine TMB). At the end, the reaction was stopped by sulfuric acid, and then the optical density was read at 450 nm wavelength against a reference of 620 nm wavelength using ELISA reader (BioTek, Epoch2, USA). Controls were included in each assay. Samples were considered positive if their values were above the cut-off value, while considered negative if they gave values lower than the cut-off. Samples with OD values in the equivocal range were retested, if they remained borderline, they were reported negative. Manufacture's instructions regarding cut-off calculation, sample handling, and storage were accurately followed.

3.4. PMNCs Separation

Ficoll-Paque PLUS (GE Healthcare Life Sciences, USA) was used for the isolation of lymphocytes from blood samples collected in EDTA tubes as per manufacturer's instructions. Briefly, 1x Hank's balanced salt solution (Life Technologies, USA) was used for 1:1 dilution of the blood samples and the diluted blood was layered on the Ficoll-Paque PLUS solution. After centrifugation, blood cells differentially migrated, resulting in the formation of different layers containing different cell types (Figure 2). The layer in the bottom contained aggregated erythrocytes, and the buffy coat containing polymorphonuclear cells (PMNCs), including lymphocytes, were at the interface between Ficoll reagent and the plasma. Plasma was discarded and PMNCs were collected in a separate tube. Finally, PMNCs were subjected to short washing steps using a balanced salt solution to remove any platelets, Ficoll-Paque PLUS and remaining plasma. Isolated PMNC were stored at -20 ^{o}C until performing DNA extraction

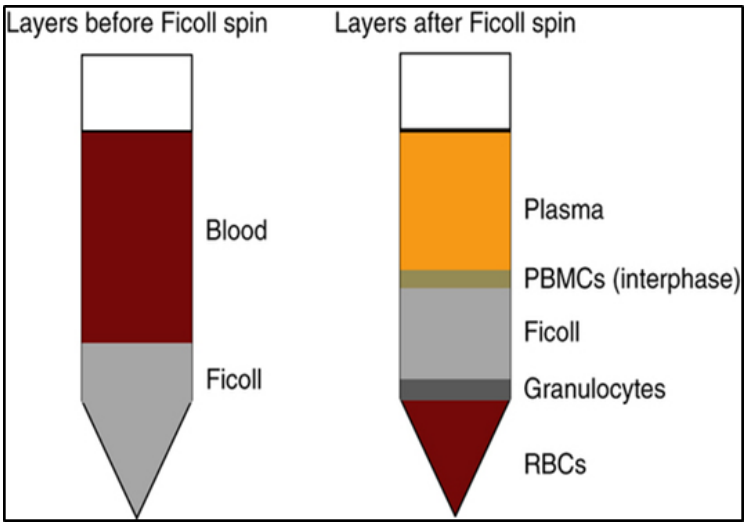

Figure 2. Ficoll separation of PMNCs. After centrifugation, blood cells differentially migrate, resulting in the formation of different layers containing different cell types . PBMCs are found in the interphase between the upper plasma layer and the ficll lower layer. . Adapted from Lin et al.,2014, Nature Protocols.

3.5 DNA Extraction

DNA was extracted from PMNCs (buffy coat samples) as per manufacturer's instruction of the DNA extraction kit (Catalog # 51106, Qiagen, Germany). The concentration and the purity of all extracted DNA were measured using NanoQuant microplate reader (Infinite pro200, Tecan, Switzerland). Extracted DNA samples were then stored at -20 ^{o}C for further testing.

3.6 EBV DNA Detection by Real Time PCR

Detection and copy number quantification of EBV DNA in all extracted samples were performed using a real-time PCR detection and quantification kit (Catalog # V48-100FRT, Sacace, Italy) as per manufacturer's instructions. The principle of the detection in this kit is based on using a real time amplification with fluorescent reporter dye probes specific for EBV. This kit is also provided with an indigenous internal control (IC), that amplifies β-globine gene, which allows for controlling the PCR amplification step, and insures the adequacy of sample preparation, materials, and storage conditions. In this kit, DNA samples were amplified using specific primers and polymerase (TaqF). The amplified DNA products were then detected using fluorescent dyes that are attached to oligonucleotide probes, which bind specifically to the DNA amplified product in the thermocycling step. QuantStudio™ 6 Flex Real-Time PCR instrument (Applied Biosystems, USA) was used for detection of the fluorescent dyes. Calibrators were used to construct the standard curve that was used to quantify EBV copies in the tested samples. The reaction was considered valid only if quantity of IC was more than 2000 copies per reaction, and no amplification is detected in the negative control.

3.7 EBV Genotyping by Nested PCR of the EBNA-2 Gene

EBV genotyping was performed using nested PCR of the EBNA2 gene, which is the target gene permitting genotype classification (8). Briefly, in the first round of amplification, primers E2p1 and E2p2 were used to amplify a fragment of 596 bp covering almost the entire EBNA2 gene (E2p1: 5'-AGGGATGCCTGGACACAAGA-3' and E2p2: 5'-TGGTGCTGCTGGTGGTGGCAA T-3'). In the second round of amplification, primers Ap1 and Ap2 (Ap1: 5'- TCTTGATAGGGATCCGCTAGGATA-3'; Ap2: 5'-ACCGTGGTTCTGGACTATCT-GGATC-3'), were used to amplify a 497 bp fragment which identify the EBV type-1 EBNA2 gene product, whereas primers Bp1 and Bp2 (Bp1:5'-CATGGTAGCCTTAGGACATA-3'; Bp2:5'-AGACTTAGTTGATGCCCTAG-3') amplified a 150 bp fragment that characterize EBV type-2 EBNA2 gene product. PCR reaction was prepared in 0.2 ml microcentrifuge tubes. In a final mixture volume of 50 µl using HotStarTaq DNA Polymerase kit (Catalog # 203203, Qiagen, Germany). Reaction mix contained 0.25 µl of HotStarTaq DNA Polymerase, 10 µl of 5x Q-Solution, 5 µl of 10x PCR buffer which already contains 15mM $MgCl_2$, all from Qiagen kit, and 1 µl of 10mM dNTP's (Catalog # N0447S, New England Biolabs, USA). Additionally, 1 µl of 10µM/ µL of the above mentioned primers. For the first round of PCR using E2p1 and E2p2 primers, amplification conditions were as follows: after an initial heat activation step of 15 min at 95 ^{o}C, 40 cycles of amplification were performed: denaturation for 5 min. at 95 ^{o}C, annealing for 1 min. at 58 ^{o}C, and extension for 1 min. at 72 ^{o}C, followed by a final extension step of 10 min. at 72 ^{o}C. For the second round of PCR, the same amplification conditions were used except for the annealing temperature: 63 ^{o}C for Ap1 and Ap2 primers, and 53 ^{o}C for Bp1 and Bp2

primers. Afterwards, amplification products were visualized on 2% agarose gel by electrophoresis. In all experiments, a negative control (sterile H_2O instead of DNA) and a positive control (previous positive sample) were used.

3.8 EBV Sub Genotyping by Sequencing of LMP-1 gene

3.8.1 Nested PCR of LMP-1 gene

For the same genotyped samples, EBV subtyping was performed using LMP-1 gene, which has a high degree of polymorphism and allows for further subgenotype identification (3). Starting with nested PCR of LMP-1 gene, the first round of amplification, primers A1 and A2 were used to amplify a fragment of 602 bp covering the C-terminus of LMP-1 gene (A1: 5'-AGTCATAGTAGCTTAGCTGAA-3'; A2 5'-CCATGGACAACGACACAGT -3'). In the second round of amplification, primers B1 and B2 aimed at amplifying a 587 bp fragment (B1: 5'-AGTCATAGTAGCTTAGCTGAA-3' ; B2: 5-AGTCATAGTAGCTTAGCTGAA-3'). Were used. PCR reaction was prepared in 0.2 ml microcentrifuge tubes to a final mixture volume of 50 µl using HotStarTaq DNA Polymerase kit (Catalog # 203203, Qiagen, Germany). Reaction mix contained 0.25 µl of HotStarTaq DNA Polymerase, 10 µl of 5x Q-Solution, 5 µl of 10x PCR buffer which already contains 15mM $MgCl_2$, all from Qiagen kit, and 1 µl of 10mM dNTP's (Catalog # N0447S, New England Biolabs, USA). Additionally, 1 µl of 20µM/ µL of the above mentioned primers was used, Amplification conditions were as follows: Starting with an initial heat activation step of 15 min at 95 ^{o}C, 40 cycles of amplification were performed in both round of PCR amplifications: 5

min. at 95 ^{o}C, 1 min. at 53 ^{o}C and 1 min. at 72 ^{o}C, followed by a final extension step of 10 min. at 72 ^{o}C. Afterwards, amplification products were visualized and analyzed using 2% agarose by gel electrophoresis. In each experiment, a negative control (sterile water instead of DNA) and positive control (previous positive sample) were used.

3.8.2 Cloning

LMP-1 PCR products from 50 randomly selected samples were cloned, sequenced and compared to previous sequences deposited in GenBank and used for phylogenetic analysis. The PCR products (587 bp) of the randomly piched samples were purified using PCR purification kit (Qiagen; Germeny). Afterwards, these PCR purified samples were exposed to TA-cloning and ligation into pDrive cloning plasmid using the QIAGEN® PCR Cloning kit, all following the manufacturers' instructions (Figure 3). Three µL of the ligated plasmids were then transformed by electroporation into 50 µL home-made *E. coli* DH5α electrocompetent cells (Invitrogen, USA). Transformed cell were then resuscitated for 30 min in 300 µL SOC broth (Invitrogen) at 37 ^{o}C with shaking. SOC bacterial suspensions were then spread on LB medium containing 100 µg/mL ampicillin (Sigma, USA). Following an incubation period for 24 h, several distinct colonies from each culture plate were randomly picked. Then, each colony was subcultured in LB containing 100 µg/ml ampicillin. After 24 hours incubation with shaking, plasmids were purified using a QIAprep® Spin Miniprep Kit (Catalog # 231122, Qiagen, Germeny).

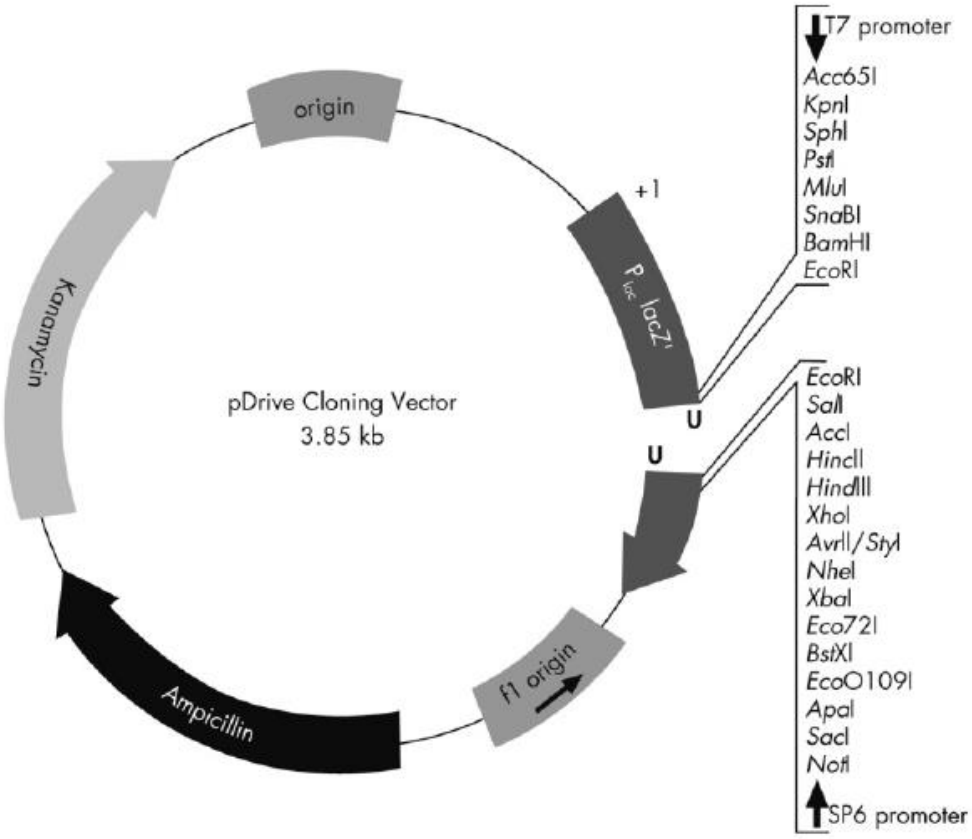

Figure 3. Cloning of the LMP-1 PCR product into pDrive cloning vector. Adapted from (Qiagen cloning kit insert sheet)

3.8.3 Phylogenetic Analysis

Prior to sequencing, amplicons were examined for the presence of the cloned PCR products using restriction digestion with EcoRI enzyme. EcoRI restriction enzyme (Catalog # R0101S, New England Biolabs, USA), and 10X EcoRI buffer (Catalog# B0101S, New England Biolabs, USA) were used. A mix of 1 µL of EcoRI enzyme, 5 µL of 1X EcoRI buffer, and 4 µL of Plasmid DNA was prepared and incubated for at least 4 hours at 37 ^{o}C. The amount of DNA in the reaction was increased or decreased based on the concentration. Digested samples were after that analyzed on 2% agarose gel by electrophoresis. Plasmids harboring the cloned fragment showed the presence of 587 bp band and a thicker large plasmid band.

Sequencing was performed using a forward primer T7 and a reverse primer Sp6, using the ABI 3730XL sequencer in a sequencing facility. MEGA, version 6.0 freeware and CLC Main Workbench version 6.0.2. (CLC; Aarhus, Denmark) was used to do sequence alignments, and to construct phylogenetic and molecular evolutionary analyses. Moreover, to assess the degree of similarity between any two sequences, pair-wise distance differences analysis was used. For sequence homology comparison, reference sequences representing reported EBV strains were used. Sequences were aligned and phylogenetic tree was generated using the neighbor joining method.

3.9 Statistical Analysis

For determining the significance relation between variable ratios , chi-square test was used. For the correlation between EBV serology and real time PCR results, Kruskal Wallis test and Mann–Whitney *U* test were used. Results were considered statistically

significant when the calculated p-value < 0.05. The statistical software SPSS.23 was used for data analysis. GraphPad Prism 7.00 software was also used for statistic tests and constructing figures.

4- RESULTS

4.1 Seroprevalence of EBV among Healthy Blood Donors in Qatar

General Characteristics of studied population: A total number of 673 blood donor samples were used in the present study. From the total tested samples, 659 (97.9%) were males, whereas 14 (2.1%) were females. The number of Qatari individuals was 223 (33.1%), and the non- Qatari residents 450 (66.9%). The age of the participant was ranged between 19 and 68 years, with a mean age of 37 years (Table 1, Figures 4, 5, 6).

For determining the seroprevalence of EBV in healthy blood donors in Qatar, all (673) plasma samples were tested for the presence of any of the following anti-EBV antibodies: VCA- IgG, VCA- IgM, EBNA1-IgG, EBNA-1 IgM, EA-IgG. We found that, 97.9% (659/673) of the tested samples were seropositive for at least one of the aforementioned anti-EBV antibodies, indicating a prior exposure to EBV (Table 2, Figure7). For the EBNA-1 IgG 616 (91.5%) tested positive, while for the IgM class against EBNA 59 (8.8%) were positive. Seroprevalence of EA-IgG was 10.5% detected in 71 of the tested samples. VCA- IgG was positive in 659 (97.9%), while 12 (1.8%) were seropositive for VCA-IgM (Table 3, Figure 8).

Serology patterns were classified into six possible EBV infection stages: Early infection, active infection, recent active infection, past infection, reactivation, and no infection. Out of the 659 seropositive individuals, 579 (86%) were positive for VCA-IgG and EBNA-IgG, or positive for VCA- IgG only, indicating past infection. Suspected viral reactivation was detected in 65 (9.7%) of samples, suggested by the positive results for VCA-IgG, EBNA-IgG, and EA-IgG. Moreover, 6 (0.89%) donors had a serologic profile that is suggestive of active infection as they tested positive for VCA-IgG, and EA-IgG,

with or without the presence of VCA-IgM. Recent active infection was suspected when VCA-IgM, VCA-IgG and EBNA-IgG test positive, which was detected in 5 (0.74%) samples. Further, 4 (0.6%) tested positive for VCA-IgM and VCA-IgG, suggesting early phase of EBV infection. Interestingly, 14 (2.1%) donors tested negative for all anti-EBV antibodies, indicating no previous or current infection (Table 4, Figure 9)

4.2 Association of EBV Seroprevalence with Age, Gender, and Nationality

In order to know whether there is an association between EBV seroprevalence and gender, age, and geographic origin of the participants, statistical analysis was performed. With regard to gender, although all females were EBV seropositive (100%), compared to the males (97.7%) positive, yet, this result doesn't show a statistical significance (p=0.58), may be due to the large difference in sample size between males (n=659) and females (n=14) (Table 2).

Qatar is a multinational country, with mixed ethnic population. Hence, we wanted to investigate EBV frequency among Qataris and non-Qatari resident, and further, among the six major nationalities that constitute the majority of our studied population. No significance was found in the EBV seroprevalence between Qataris and non- Qataris (p=0.84). Out of 233 Qatari donors, 218 (97.8%) were seropositive, and out of 450 donors from other nationalities, 441 (98%) were seropositive. Further analysis was done among six major nationalities: Qatar (n=223), Syria (n=95), Egypt (n=92), Jordan/Palestine (n=61), India (n=59), Pakistan (n=20). Egyptians and Pakistanis had the highest rate of EBV seroprevalence (100%) in both countries, followed by Indian (98.3%), Syrian (96.8%), Qatari (97.8), and Jordanian/Palestinian nationalities (95.1%).

There was no statistical significance in EBV seroprevalence, or the stage of infection between the major nationalities of the studied population (p>0.05) (Table 4 and 5).

Generally, because EBV seroconversion is age oriented (57), thus, the association between age of participants and EBV seroprevalence was investigated in this study . We found that the infection rate increased significantly with age (p= 0.03), from 96% in individuals less than 30 years old, to 100% in people more than 40 years old. All individuals older than 40 years (n=233) were EBV seropositive (Table 4 , Figure 10).

4.3 EBV Viremia Rate among Healthy Blood Donors

A total of 673 DNA extracted samples were tested for the presence of EBV DNA, and for quantification of the viral load using qRT-PCR. EBV DNA was detected in 354 (52.6%) of the samples. No significant association was found in viremia rate between males (52.8%) and females (42.9%), or between Qataris (55.2%) and non-Qataris (51.1%), or among major nationality groups. However, in regard to age, there was a significant association of viremia rate between age groups (p-value = 0.009), as viremia rate increased with age (Table 3). The lowest viremia rate was detected in the age group of people less than 30 years (44.8%), whereas the highest rate was exhibited by people aged 51 and older (67%) (Table 6 and 7).

EBV viral load was calculated in copies per reaction, and copies per one microgram of extracted DNA. Viral load in the positive samples was ranged from 0.24 copies/µg of DNA to 873.24 copies/µg, with a mean of 27.89 copies/µg.

4.4 Correlation between Serology and Real Time PCR findings

For the total 673 tested samples, the correlation of serological testing by ELISA, and the EBV DNA detection by real time PCR was investigated. All EBV seronegative donors who had negative ELISA results for the five EBV antibodies, showed undetectable EBV DNA in their blood 14/14 (100%). Moreover, all donors with a antibodies profile suggesting EBV active infection had amplifiable EBV DNA 6/6 (100%). However, 3 out of 4 donors that were classified with an early EBV infection had positive EBV PCR (75%). For EBV recent active infection category, 2 out of 5 (40%) only showed detectable EBV DNA by PCR. And in EBV reactivation, 39 samples out of 65 (60%) were EBV PCR positive. Last, 36/65 (52.6%) of individuals with EBV past infection had detectable levels of EBV DNA in their blood (Table 8, Figure 11).

The correlation between EBV viral load with the stage of infection was investigated. There was no significant difference in the viral load between different EBV stages ($p>0.05$) (Figure 12).

4.5 EBV Genotypes among Blood Donors

A total of 51 randomly selected samples from different nationalities were genotyped, using primers that amplify the EBNA2 gene. After the first round of PCR, all EBV positive samples produced a 597 bp fragment of the EBNA2 gene (Figure 13).

Then, EBV type specific primers were used to distinguish between type 1, and type 2. EBV type 1 was characterized by an amplicon of 479 bp in length when using AP primers, while EBV type 2 was identified by a PCR product of 150 bp using BP primers (see materials and methods page 31). In samples with mixed EBV infection, both sets of

primers AP and BP positively amplified a 479 bp and 150 bp fragments respectively (Figure 14 and 15).

Results of EBV genotyping showed that EBV type 1 is predominant. Out of the 51 genotyped samples, there were 37 (72.5%) donors infected with EBV type 1, 12 (23.5%) with type 2, and 2 (3.9%) with a mixed infection of both EBV genotypes. No significant association was found between EBV types and nationalities ($p>0.05$), and EBV type 1 was more prevalent than EBV type 2 in all nationalities (Table 9)

4.6 EBV Strains by Sequencing of the LMP-1 gene

Out of the 51 genotyped samples, 30 samples were selected used for further sub genotyping of EBV into different subgenotypes or strains. To do that, the C-terminus of the LMP-1 gene was amplified using nested PCR (see materials and method section, page 32). The product of the second PCR round, which amplified a 587 bp fragment was cloned in the multiple cloning site of the pDrived cloning plasmid. Positive clones were tested for the presence of the cloned fragment using EcoRI restriction digestion (Figure 16 and 17). Three to six clones from each samples (total of 119 clones) were sent to MC Lab for sequencing.

The sequences of the EBV LMP1 gene were aligned and compared to the reference sequences of previously reported EBV strains: The prototype B95-8, Med+, Med- , China 1, China 2, Alaskan, NC, CAO and SEA 1 (CD+16). Molecular Phylogenetic analysis by Maximum Likelihood method was performed (Figure 18). Surprisingly, analysis revealed the dominance of CD+16, also known as South Eastern

Asia 1 (SEA1) strain, which is a close derivative that belongs to China 1 strain. All of the sequenced clones 119/119 (100%) were carrying CD+16 (SEA1) strain.

4.7 LMP-1 30 bp and 69 bp Deletions

Due to high variability of the LMP-1 gene, mutational hot spots and deletions can be found. Therefore, we have examined the presence of two previously reported deletions (del- LMP1): 30 bp deletion, and a larger deletion of 69 bp in length. Our results shows that 3/30 (10%) of the samples had 30-bp deletion. In addition, 4/30 (13.3%) had the 69 bp deletion. All of the aforementioned deletions were within EBV type 1 samples. No deletions were detected in the 5 sequenced samples that belong to EBV type 2 (Table 10).

Table 1. *Demographic Characteristics of the Studied Subjects (N=673)*

Category		No.(%)
Gender		
	Male	659 (97.9)
	Female	14 (2.1)
Nationality		
	Qatari	**223 (33.1)**
	Male	219 (98.2)
	Female	4 (1.8)
	Non-Qatari	**450 (66.9)**
	Male	440 (97.8)
	Female	10 (2.2)
Age		
	<20-30	176 (26.15)
	31-40	264 (39.2)
	41-50	174 (25.9)
	>50	59 (8.8)

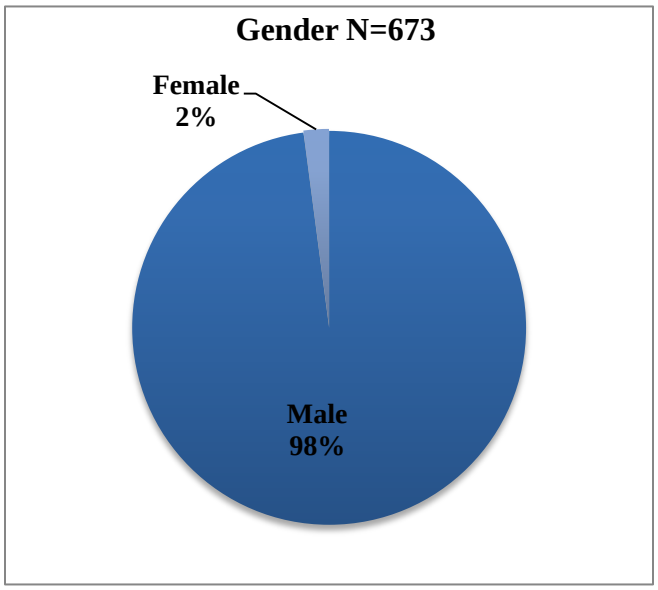

Figure 4. Gender distribution of the studied subjects. Total number =673

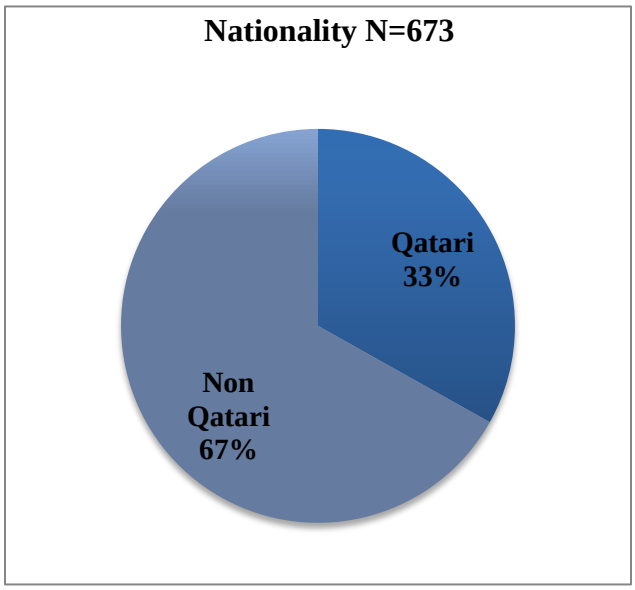

Figure 5. Nationality distribution of the studied subjects. Total number = 673

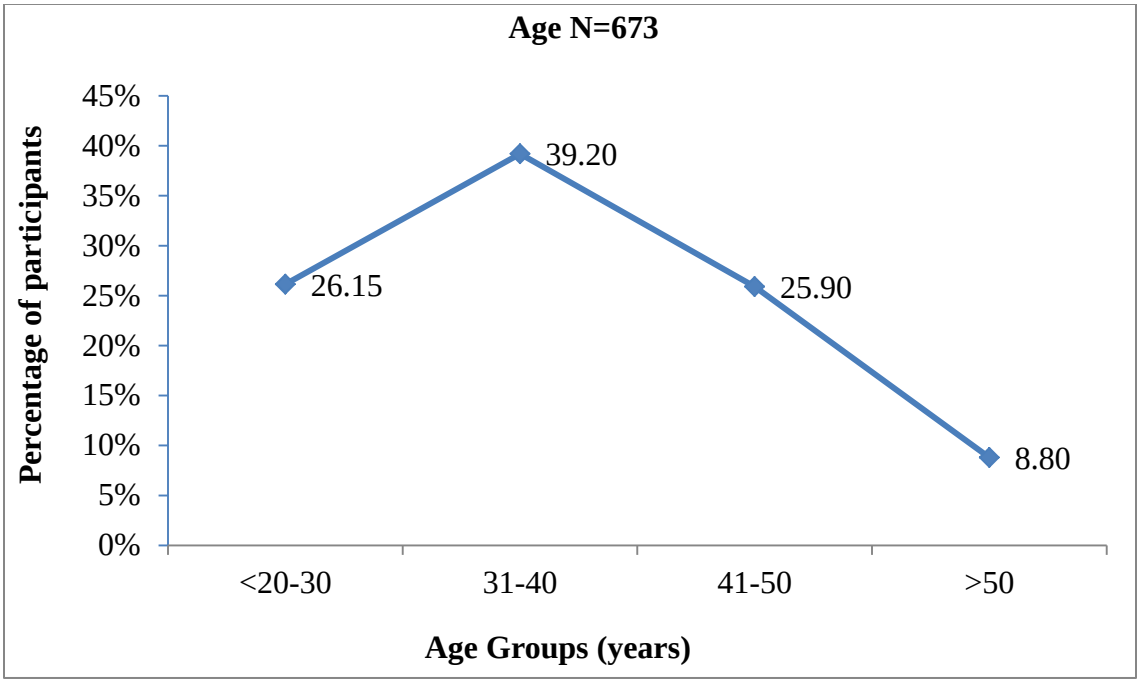

Figure 6. Distribution of age groups in the studied population. Total number =673. Mean age =37.1, SD=9.4.

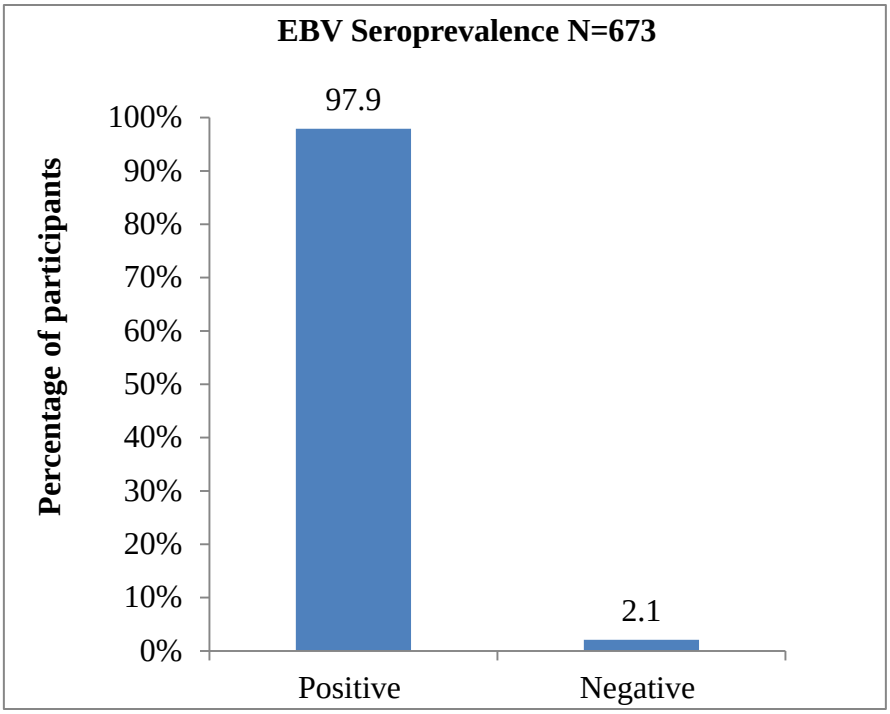

Figure 7. EBV seroprevalence in the studied subjects defined by a one or more positive reaction for EBV antibodies: VCA-IgG, VCA-IgM, EA-IgG, EBNA-IgG, and EBNA-IgM.

Table 2. *EBV seroprevalence in the studied population (N=673)*

Category		Total No.	EBV serology		*P* -Value
			Positive No. (%)	Negative No. (%)	
Gender					
	Male	659	645 (97.9)	14 (2.1)	0.581
	Female	14	14 (100)	0 (0)	
Nationality					
	Qatari	**223**	218 (97.8)	5 (2.2)	
	Male	219	214 (97.7)	5 (2.3)	
	Female	4	4 (100)	0 (0)	0.8358
	Non-Qatari	**450**	441 (98)	9 (2)	
	Male	440	431 (97.9)	9 (2)	
	Female	10	10 (100)	0 (0)	
Age Group					
	<20 - 30	176	169 (96)	7 (4)	
	31 - 40	264	257 (97.3)	7 (2.7)	0.037
	41 - 50	174	174 (100)	0 (0)	
	> 51	59	59 (100)	0 (0)	

Table 3. *Seroprevalence of different EBV antibodies in the studied population (N=673)*

EBV antibody	No of positive samples (%)
VCA- IgM	12 (1.8)
VCA- IgG	659 (97.9)
EA - IgG	71 (10.6)
EBNA - IgM	59 (8.8)
EBNA - IgG	616 (91.5)

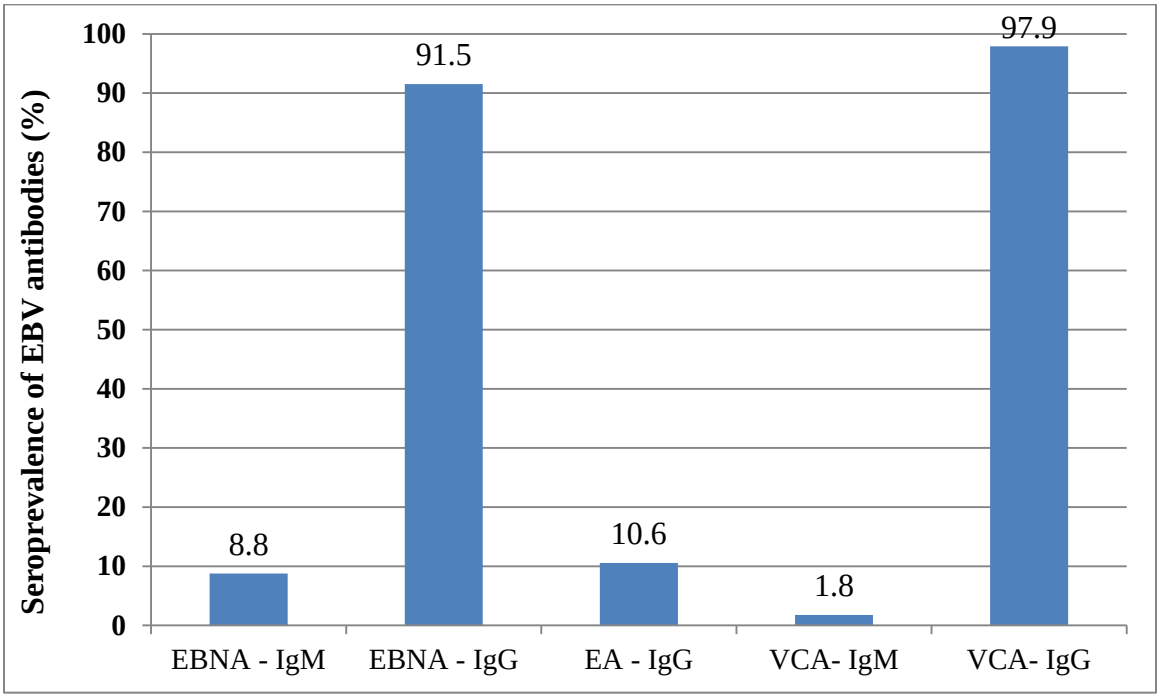

Figure 8. Seroprevalence of EBV antibodies: EBNA-IgM, EBNA-IgG, EA-IgG, VCA-IgM, and VCA-IgG, among the studied subjects. Detection of EBV antibodies in the plasma samples was performed by ELISA technique.

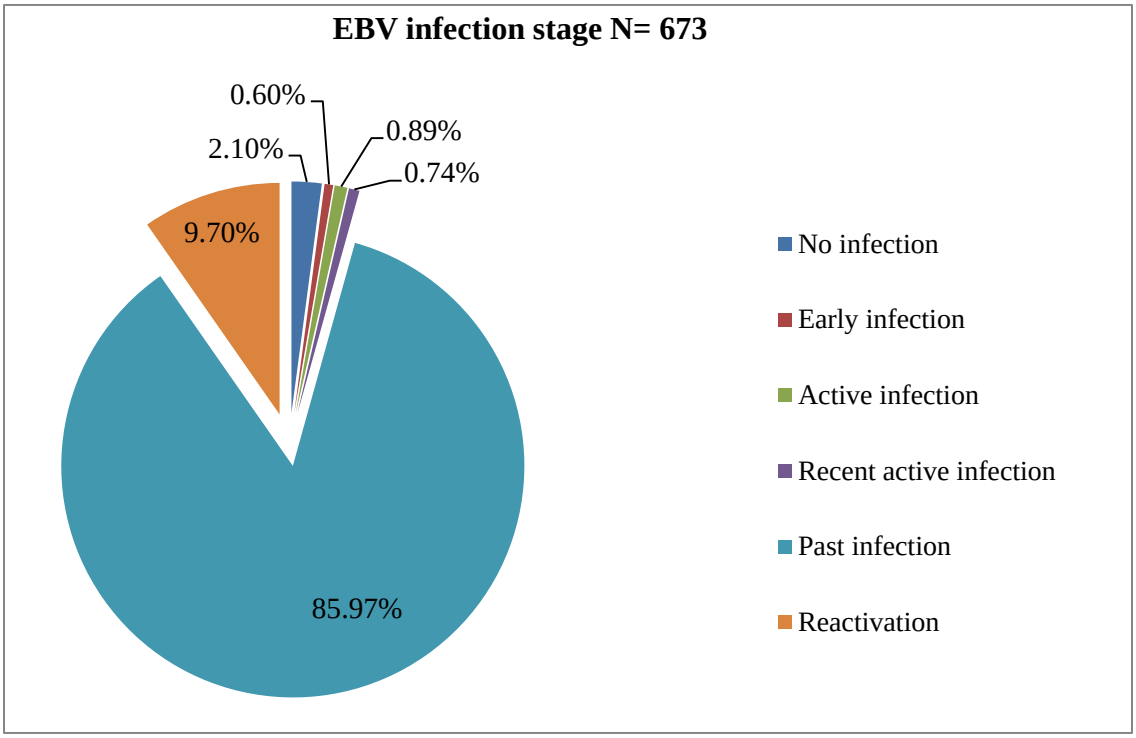

Figure 9. EBV presumed stage of infection according to the ELISA results. Serology patterns were classified into six possible EBV infection stages: Early infection (positive VCA-IgM and VCA-IgG), active infection (positive VCA-IgG, and EA-IgG, with or without VCA-IgM), reactivation (positive VCA-IgG, EBNA-IgG, and EA-IgG), recent active infection (positive VCA-IgM, VCA-IgG, EBNA-IgG). No infection (negative for all anti-EBV antibodies)

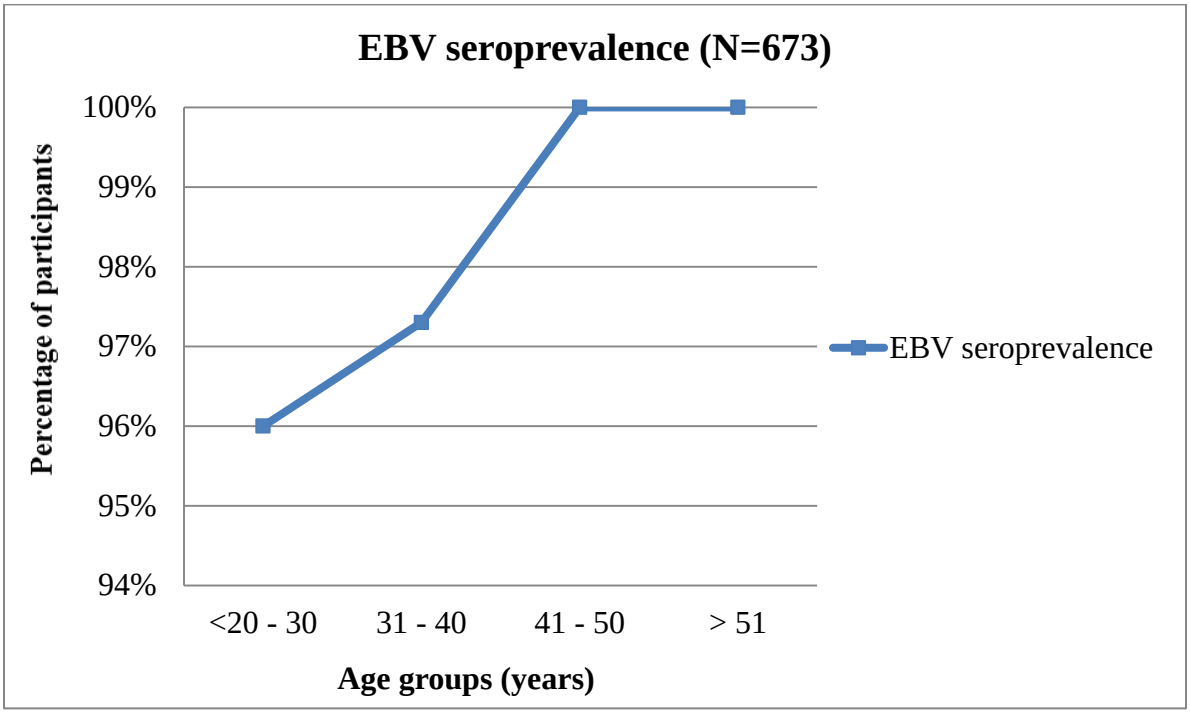

Figure 10. EBV seroprevalence in different age groups tested by ELISA. Seroprevalence ranged from 96% in the youngest age group (<20-30 years), to 100% in the eldest age group (>51 years).

Table 4. *EBV infection stage related to gender, nationality, and age group (N=673)*

		EBV serology											
		Past infection		Early infetion		Active Infection		Recent Active		Reactive infection		No infection		P-Value
Category	Total No.	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)	
Gender														
Male	659	572	(86.6)	3	(0.5)	6	(0.9)	5	(0.8)	59	(9)	14	(2.1)	<0.001
Female	14	7	(50)	1	(7.1)	0	(0)	0	(0)	6	(42.9)	0	(0)	
Nationality														
Qatari	223	191	(85.7)	1	(0.4)	1	(0.4)	2	(0.9)	23	(10.3)	5	(2.2)	0.949
Non-Qatari	450	388	(86.2)	3	(0.7)	5	(1.1)	3	(0.7)	42	(9.3)	9	(2)	
Age Group														
<20 - 30	176	146	(83)	2	(1.1)	2	(1.1)	2	(1.1)	17	(9.7)	7	(4)	0.337
31 - 40	264	230	(87.1)	2	(0.8)	2	(0.8)	2	(0.8)	21	(8)	7	(2.7)	
41 - 50	174	150	(86.2)	0	(0)	2	(1.1)	0	(0)	22	(12.6)	0	(0)	
> 51	59	53	(89.8)	0	(0)	0	(0)	1	(1.7)	5	(8.5)	0	(0)	
Total	673													

Table 5. *EBV seroprevalence among major nationalities (N=673)*

Category		Total No.	EBV Serology		*P* -Value
			Positive No. (%)	Negative No. (%)	
Nationality					
	Qatar	223	218 (97.8)	5 (2.2)	0.4
	Syria	95	92 (96.8)	3 (3.2)	
	Eygpt	92	92 (100)	0 (0)	
	India	59	58 (98.3)	1 (1.7)	
	Jordan/Palest	61	58 (95.1)	3 (4.9)	
	Pakistan	20	20 (100)	0 (0)	
	Others	123	121 (98.4)	2 (1.6)	
	Total	673			

Table 6. *EBV viremia rate among the studied subjects (N=673)*

Category		Total No.	EBV DNA		*P* -Value
			Positive No. (%)	Negative No. (%)	
Gender					
	Male	659	348 (52.8)	311 (47.2)	0.317
	Female	14	6 (42.9)	8 (57.1)	
Nationality					
	Qatari	**223**	123 (55.2)	100 (44.8)	0.323
	Male	219	112 (51.1)	98 (44.7)	
	Female	4	2 (50)	2 (50)	
	Non-Qatari	**450**	230 (51.1)	220 (48.9)	
	Male	440	227 (51.6)	213 (48.4)	
	Female	10	4 (40)	6 (60)	
Age Group					
	<20 - 30	176	79 (44.8)	97 (55.1)	0.007
	31 - 40	264	134 (50.76)	130(49.2)	
	41 - 50	174	101 (58)	73 (41.9)	
	> 51	59	40 (67)	19 (32.2)	

Table 7. *EBV viremia rate among major nationalities (N=673)*

Category		Total No.	EBV DNA		*P* -Value
			Positive No. (%)	Negative No. (%)	
Nationality					
	Qatar	223	123 (55.2)	100 (44.8)	0.6
	Syria	95	51 (53.7)	44 (46.3)	
	Eygpt	92	47 (51.1)	45 (48.9)	
	India	59	28 (47.5)	31 (52.5)	
	Jordan/Palest	61	28 (45.9)	33 (54.1)	
	Pakistan	20	13 (65)	7 (35)	
	Others	123	64 (52)	59 (48)	
	Total	673			

Table 8. *EBV viremia rate in different infection stages*

Category by ELISA	Total No	EBV detection by RT-PCR		*P*- value
		Positive No. (%)	Negative No. (%)	
No infection	14	0 (0)	14 (100)	<0.001
Early infection	4	3 (75)	1 (25)	
Active infection	6	6 (100)	0 (0)	
Recent active infection	5	2 (40)	3 (60)	
Past infection	579	304 (52.5)	275 (47.5)	
Reactivation	65	39 (60)	26 (40)	
Total	673	354	319	

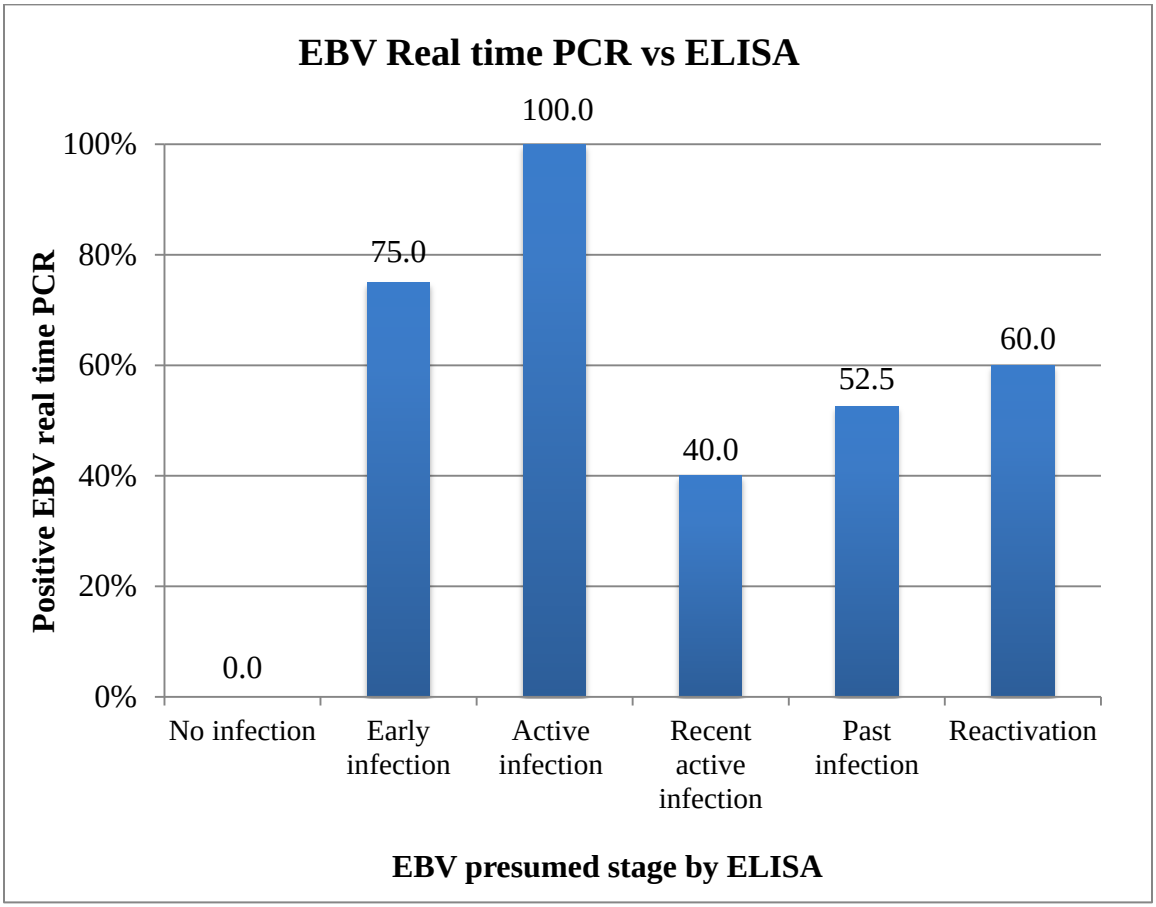

Figure 11. EBV viremia rate in different infection stages. EBV DNA was detected using real time PCR for the DNA extracted from buffy coat. Serological patterns classified by serological testing of EBV antibodies using ELISA: Early infection (positive VCA-IgM and VCA-IgG), active infection (positive VCA-IgG, and EA-IgG, with or without VCA-IgM), reactivation (positive VCA-IgG, EBNA-IgG, and EA-IgG), recent active infection (positive VCA-IgM, VCA-IgG, EBNA-IgG). No infection (negative for all anti-EBV antibodies).

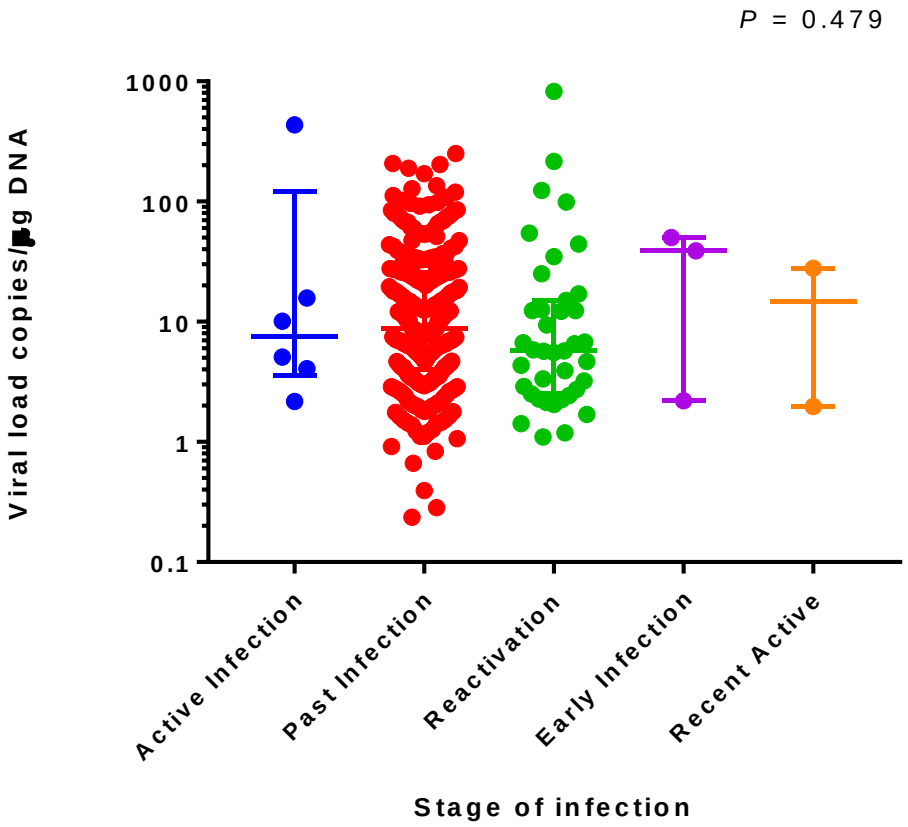

Figure 12. Correlation between EBV presumed stage and EBV DNA viral load (in copies per µg of DNA). EBV viral load was quantified using quantitative real time PCR. Serological patterns were classified using ELISA testing.

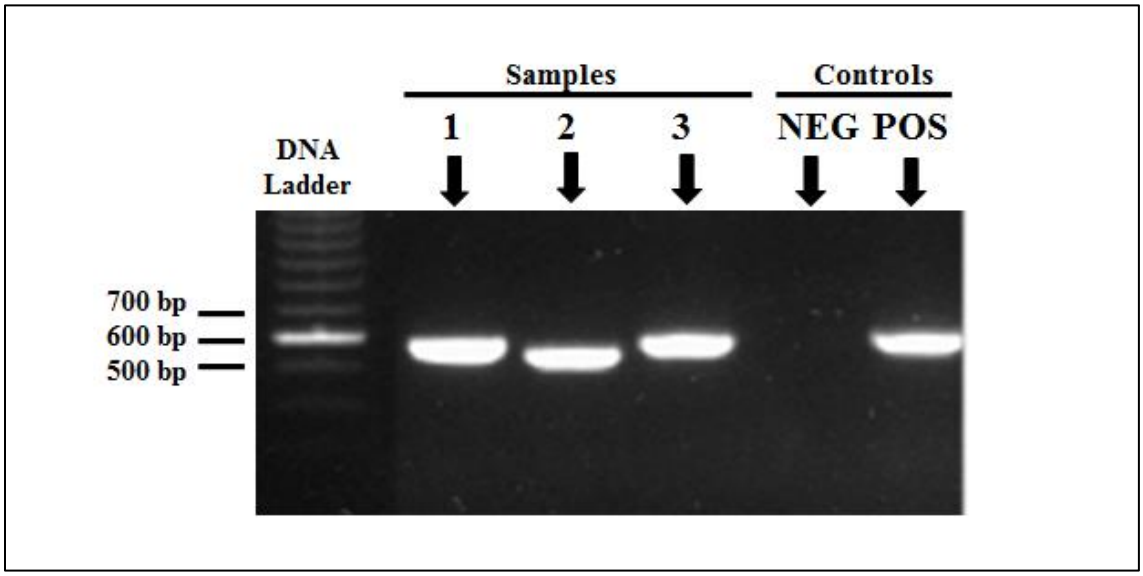

Figure 13. The first round of PCR amplification for the EBNA2 gene using E2P1 and E2P2 primers that amplified almost the entire EBNA2 gen: 597 bp in length (Lane 1, 2 and 3). Positive control: EBV positive samples. Negative control: sterile water.

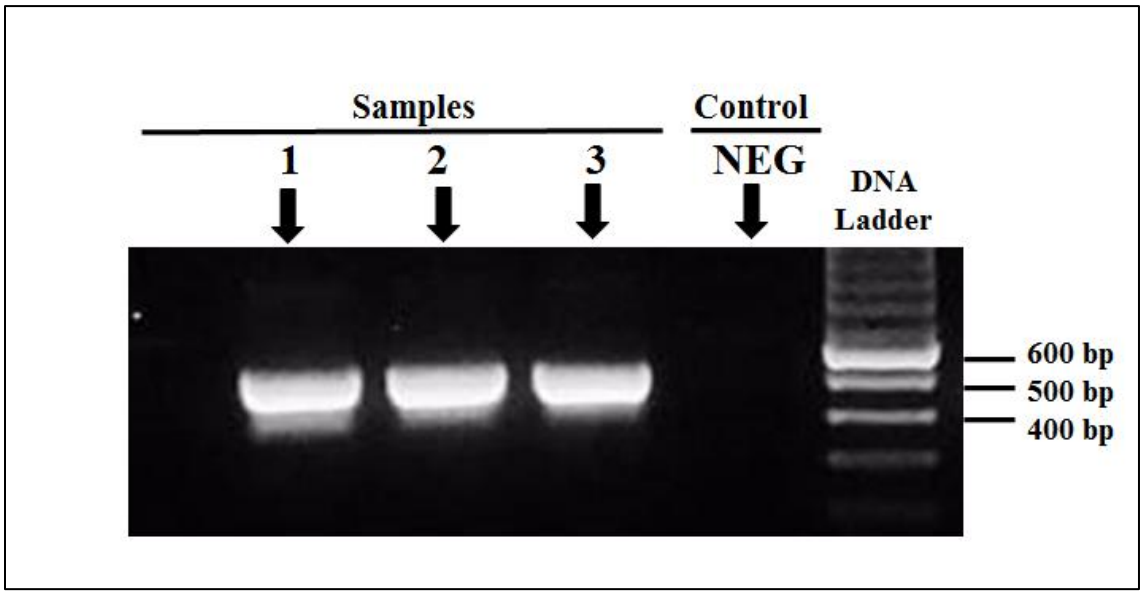

Figure 14. The second round of PCR amplification for the EBNA2 gene using EBV type 1 specific primers (AP primers). Genotype 1 samples amplify a 497 bp fragment (Lane 1,2, and 3). Sterile water was used as negative control.

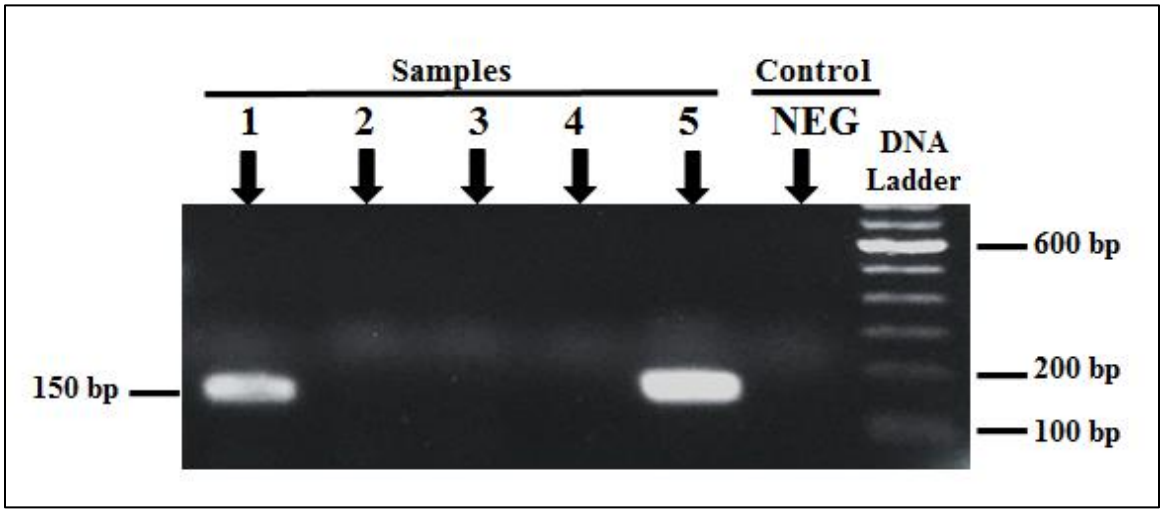

Figure 15. The second round of PCR amplification for the EBNA2 gene using EBV type 2 specific primers (BP primers). Genotype 2 samples amplified a 150 bp fragment (Lane 1, and 5). Sterile water was used as negative control

Table 9. *Description of EBV genotypes (N=51)*

No. of EBV genotyped samples	No. (%)
Genotype 1	**37/51 (72.5%)**
Qatari	10/14 (71.4)
Non-Qatari	27/37 (72.9)
Genotype 2	**12/51 (23.5 %)**
Qatari	3/14 (21.4)
Non-Qatari	9/37 (24.3)
Mixed Genotype 1 & 2	**2/51 (3.9%)**
Qatari	1/14 (7.1)
Non-Qatari	1/37 (2.7)

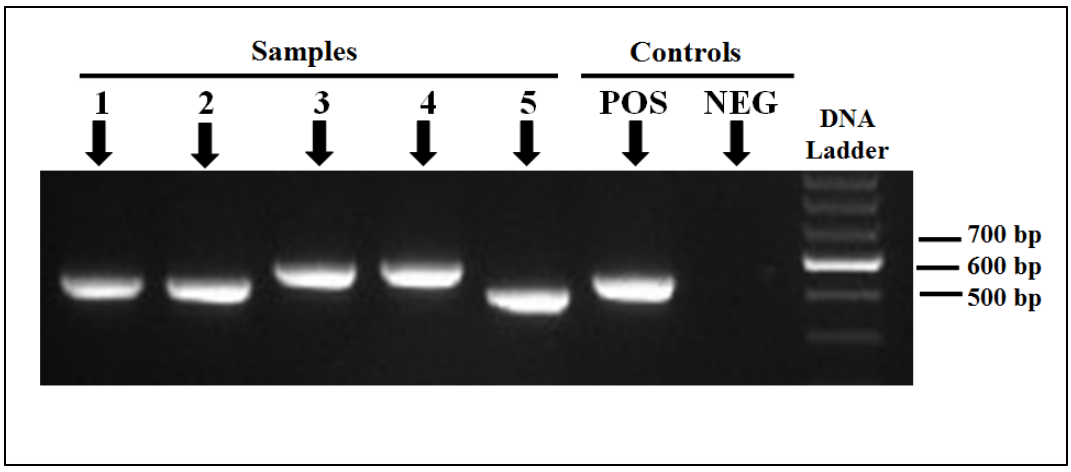

Figure 16. PCR amplification of the C- terminus of LMP-1 gene using primers that amplify a 587 bp fragment.

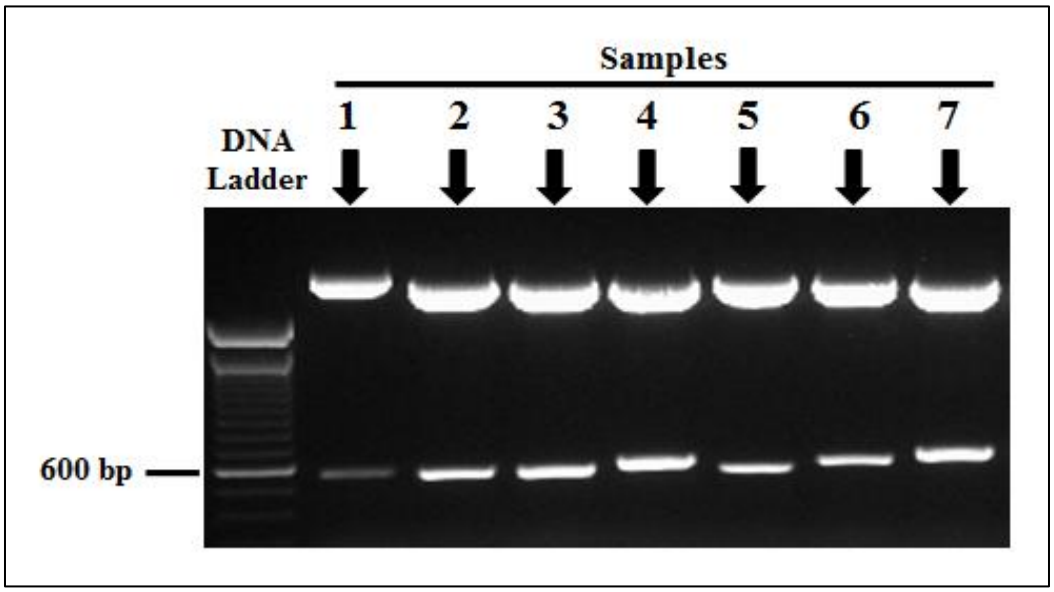

Figure 17. EcoRI restriction digestion of the pDrive cloning plasmids harboring the LMP-1 PCR fragment. The 587 bp cloned fragments (Lane 1-7) were dropped out after digestion of the pDrive plasmid (upper thick band) with EcoRI.

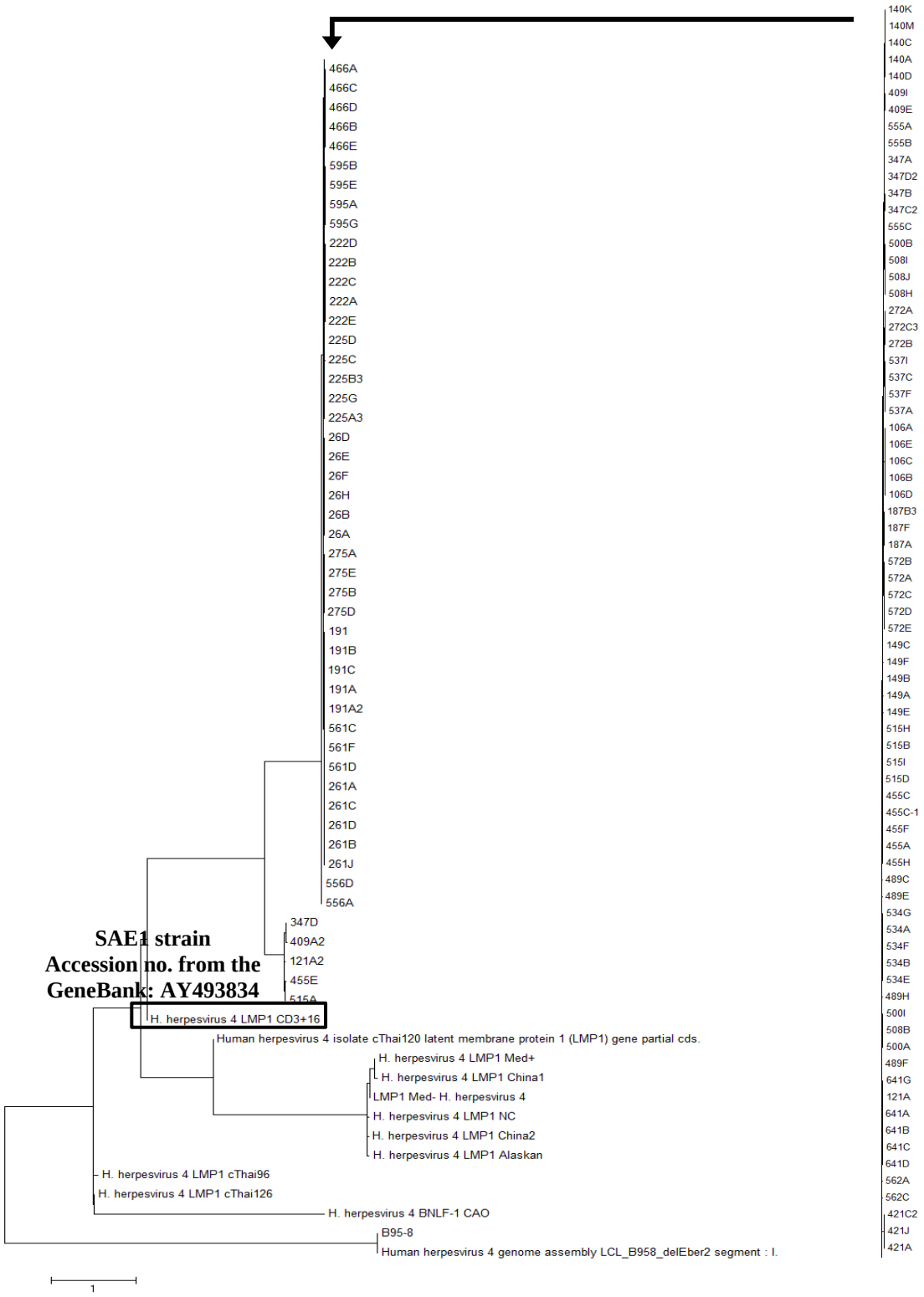

Figure 18. Phylogenetic tree of LMP-1 gene sequences. Pair-wise distance differences analysis was used and reference sequences representing reported EBV strains were used for sequence homology comparison. Sequences were aligned and phylogenetic tree was generated using the neighbor joining method.

Table 10. *Summary of Deleted and Non-Deleted Isolates (n=30)*

EBV genotype	Total no.	30 bp deletion (%)	69 bp deletion (%)	non deleted (%)	*p*- value
Type 1	25	3 (12)	4 (16)	18 (72)	0.609
Type 2	5	0 (0)	0 (0)	5 (100)	
Total	30	3 (10)	4 (13.3)	(23) 76.7	

5- DISCUSSION

In this study, EBV seroprevalence among healthy blood donors was found to be 97.9%, which is close to the EBV general seroprevalence rate around the world. Although socioeconomic factors contribute to the age of EBV seroconversion, yet, in adulthood, 90 to 99% of individuals, are usually EBV seropositive (17, 57). According to the CDC estimates, 95% of adults at age between 35 and 40 years age have been previously exposed to EBV infection (56). Our results also show that there is a significant association between EBV seroprevalence and age. In this study, seroprevalence increased with age until it reached to 100% prevalence in the group of the participants aged 40 years or older. Similar findings were reported in a study that investigated the seroprevalence of EBV in different age groups in Thailand, as EBV seroprevalence increased with age and all individuals are EBV seropositive at age of 40 years (114). In another study conducted in US to estimate EBV seroprevalence among organ donors, it revealed that 94% of donors were seropositive and higher percentage of EBV seronegative donors was observed in the young age groups (115).

When we investigated the association between EBV seroprevalence and nationality, as expected, there was no significant difference. All nationality groups had close prevalence rates. EBV seropositivity can be affected by socioeconomic factors, which vary between different geographic areas. However, at adulthood, more than 90% of people are seropositive (5, 114). As all our studied subjects are adult blood donors with a minimum age of 19 years, no significant difference in the rate of infection was found between different nationalities.

Although the indirect immunofluorescence assay (IFA) is considered the gold standard for EBV immune-diagnosis, enzyme immunoassay (EIA) is the routine diagnostic technique used because of its high throughput (78). Compared to other techniques, serological testing is still preferable and commonly used although it shows high degree of variability (73). Detection of EBV antibodies in the serum of patients can help to identify the stage of infection, but can also mislead the diagnosis or produce false results.

EBNA-1 antibodies are usually used to identify past infection as they appear late in the stage of infection, however, in our study, 6.7 % of the donors with past infection had negative result for EBNA-1 antibodies, although they are positive for VCA-IgG. Previous reports have also revealed that 5 to 10% of healthy people never develop EBNA-1 antibodies (76). Moreover, in immunosuppression, loss of anti EBNA-1 is frequently seen (85). As reported by others (84), our results confirm that the use VCA-IgG is superior to EBNA-1 IgG to indicate prior exposure to EBV, as VCA-IgG was found in all EBV seropositive individuals. Balfour et. al. reported that measuring VCA-IgG antibodies is found to be best single test to indicate a previous EBV infection (84).

Furthermore, EA- IgG is usually undetected after convalescence. When EA- IgG is detected, it might indicate reactivation of the virus (90). However, reactivation can't be confirmed by only the presence of EA-IgG. That is, several studies have shown that approximately 20% of previously infected individuals retain a positive EA-IgG for years (76). Among our tested samples, 65 (9.7%) have positive EA-IgG, and therefore, were classified under EBV reactivation category. Yet, even though EBV reactivation is not rare among healthy individuals and it can occur periodically, these findings need to be

supported by other tests, and by the clinical picture of the patient/donors to confirm a past infection with persistent EA-IgG.

Detection of EBV DNA revealed that 52.6% of our studied subjects carry EBV DNA in their peripheral blood mononuclear cells (PMNCs). Previous studies have also investigated EBV viremia among healthy subjects. Hudnall et. al reported high rate of EBV viremia among healthy blood donors. They found that 72% of 100 randomly selected donors have detectible EBV in their white blood cells (58). In another study with larger samples, Nishiwaki et. al reported that out of 953 healthy individuals, 39.5 % had EBV viremia in their PMNCs (59). A lower rate (5.1% of 198 tested samples) was reported in similar study that investigated the presence of EBV DNA in healthy individuals (60). However, in the latter study, the type of sample that was used for EBV detection was not indicated. EBV detection by PCR is highly affected by the specimen used, and the variation in sample types must always be considered when comparing different studies. Usually healthy individuals don't carry EBV in their cell free proportion of blood. Studies that detected EBV in serum of healthy individuals reported very low or undetected EBV viremia (97, 100, 106).

Since detection of EBV DNA is considered a reliable method, which complements serology testing, and aids in diagnosing acute infection and confirming silent reactivation (50, 77, 100, 101). We investigated the correlation of EBV DNA presence and ELISA results. We also investigated the correlation of ELISA results with EBV viral load. For the EBV seronegative group, our PCR results were well-matched with the serology results. All donors classified by ELISA as EBV negative showed undetectable EBV DNA by PCR. Similarly, all samples with a serological pattern of

EBV active infection carried amplifiable quantity of EBV DNA. However, discrepancies between serology and PCR were found in early infection, reactivation, and recent active infection groups.

In the group of EBV early infection, with positive VCA-IgM and VCA-IgG antibodies, 3 out of 4 samples showed positive PCR results, while one sample was PCR negative. The reason behind this could be false positive ELISA results, or the amount of EBV was below detection limit. Previous studies showed that circulating EBV DNA levels in whole blood or plasma increases during the first two weeks on infection, then declines to low or undetectable levels after several weeks to months (40). Interestingly, kinetics of EBV differs from person to person, and different levels are reached before the it became stable (116).

In EBV recent active infection group, PCR results were also not consistent with the serology results. This category is defined by positive antibodies against VCA-IgM, VCA-IgG and EBNA-IgG. However, we believe that PCR results are more reliable, due to the high rate of false positive results in VCA-IgM immunoassays (82, 83). Cross-reactivity of VCA-IgM determined was reported with other antigenically related infections, especially with CMV infection (82, 83). Moreover, the specificity of the ELISA kit we used is 93.2% as stated by the manufacturer. This indicates the possibility of having false positive results.

In samples classified with EBV reactivation, 60% were PCR positive. EBV reactivation was identified by the presence of past infection antibodies VCA-IgG and EBNA1-IgG, with detectable EA-IgG again. However, EA-IgG persists for life in around 20% of individuals (73, 76). Hence, based on serology only, reactivation can’t be

confirmed, and it can't predict the exact reactivation time (50). EBV DNA detection and viral load quantification is used to assist in the diagnosis of EBV reactivation, yet, discrepancies can be found between PCR and serology. In a study that investigated EBV reactivation in healthy carriers, Maurmann et. al reported that EBV viremia and viral load changes happen more frequently in contrast with the serological profile. Further, this could be explained due to the low sensitivity of serological assays (50).

In our study, comparison of EBV viral load in different EBV stages showed that there is no significant difference among those different groups. High viral load were expected to be found in reactivation or acute infection, but there was no significant difference. This could be explained by the difference in sample size in each group. Samples with active infection or reactivation were much less than past infection samples. This didn't allow for a valid comparison in EBV viral load. Moreover, our study is cross sectional, we tested only one sample for each donor, and we didn't follow up or test more samples for each individual in different time periods. Hence, a phase of EBV viremia or viral load increase is possible to be missed.

In the present study, EBV genotyping revealed predominance of genotype 1, which was found in 72.5% of 51 genotyped individuals. This genotype is usually more prevalent in Europe, America, China, and South Asia (5, 55). On the other hand, EBV type 2 was detected only in 23.5% of samples. EBV type 2 is less prevalent in the aforementioned populations and is more observed in African and Papua New Guinean populations. (5, 55). Recent study investigated that prevalence of EBV genotypes in different ethnic groups in Polish, Taiwanese and Arabic individuals. Results revealed that in both Polish and Taiwanese, EBV type 1 was more prevalent, it was identified in

81.8% cases, while type 2 was found in 18.2% cases. Mixed infection with both genotypes was found only in one on the two Arabic participants (33). In our study, mixed infection was also detected with low prevalence. Only 2/51 (3.9%) had both types of EBV. Previous studies also have reported that healthy individuals can acquire a mixed EBV infection (9, 117). As reported by others, because of mixed infection, it could be concluded that the one genotype does not provide immunity against the other genotype.

In investigating the subtypes of EBV, sequencing of the LMP-1 gene revealed that all our samples have SEA1 strain. This strain was previously reported in a study that identifies EBV strains in healthy and diseased individuals in Southern Thailand. Saechan et al. reported that EBV SEA1 strain was found in 1 out of 18 tested healthy individuals (11). However, to our knowledge there are no previous studies that investigated the circulating EBV strains among Qatari or other Arabic neighboring countries, and this is the first study to report the EBV LMP-1 strains among healthy individuals.

Deletions in the LMP-1 gene was correlated to the prognosis of EBV associated diseases, and some are reported in healthy individuals in previous studies (31, 33, 118). We have found that 12% of our sequenced subjects had a 30 bp deletion. Moreover, 4% had a 69 bp deletion. Our findings are supported by other studies from different populations. LMP-1 30 bp deletion was reported in healthy Polish and Taiwanese carriers in a recent study by Polz et al. (33). In another study investigated LMP-1 deletions among Moroccan patients compared to healthy individuals, they reported that about 14% of their healthy donors carry the 69 bp deletion, and 36% carried 30 bp deletion (119)

Study Limitations

This study was applicable for investigating EBV seroprevalence, genotypes, and sub genotypes among healthy individual with different nationalities and age groups. However, there are some limitations. For instance, in regard to gender, we couldn't have a valid comparison because of the large difference between female number (n=14) and males (n=659). Further, although statistical analysis revealed a significant difference in stage of infection between males and females, this significance could be considered invalid and can't be generalized. The number of donor males is usually higher than females, however, increasing the sample size can increase the number of females and allow for a better comparison. Moreover, our samples were collected from the donor unit, and the minimum age of blood donation is 18. The majority of our studied samples were between 31-40 years old, and no participants were below the age of 19. In order to study EBV seroconversion and investigate the best age of vaccine administration, it is important to include younger age groups.

Serological testing for approximately 700 sample was in fact time consuming, because each sample was tested for 5 different EBV antibodies. As a result, for all samples, a total of more than 3500 ELISA tests were performed. Despite the time and cost spent on these tests, not all samples were included in the analysis. Our sample size was corrected to 673, due to the lack of some important demographic data that we couldn't obtain from the donor unit.

VCA-IgG Avidity assay was to be performed to confirm ELISA classification in acute infection samples, especially for those with inconsistent ELISA and PCR results.

But due to the time limitation of this study and unavailability of the confirmation tests, this assay was not included in the current results, but will be performed in the near future.

Future Work

- Performing VCA-IgG avidity assay to confirm the ELISA results and to solve the inconclusive findings in comparison with PCR results
- As our participants are all healthy individuals, we used the cells proportion of the blood to detect and measure EBV viremia. Measuring EBV viral load in the plasma of acute infection donors, will aid also in the confirmation of acute infection.
- Cloning and sequencing for more samples to confirm our genotyping findings and also to study EBV strain variation in a larger sample size.
- We have performed our EBV strain variation analysis on the DNA level. We compared the nucleotide sequence with the previously reported sequences. In the future, translation of the sequenced clones will be performed, in order to study the changes in amino acid. That is, performing genotyping based on amino acid sequence could revealed more accurate results and findings of other circulating sub genotypes in this part of the world.

6- **CONCLUSION**

EBV is a highly prevalent infection, affecting more than 90% of world's population. Many studies have been done to estimate the rate of EBV seroprevalence. However, national reports in Qatar that estimate EBV prevalence are not available, and to our knowledge, this study represents the first estimate of EBV in Qatar.

We have shown than EBV seroprevalence is 97.89%, and viremia rate is 52.6%, both significantly increase with age. EBV Genotype 1 and Subtype SEA1 are the predominating (72.5%, 100%, respectively) in Qatar. Moreover, LMP-1 30 bp and 69 bp deletions were found in 10% and 13.3%, respectively. This study should provide the epidemiologists, blood banks' personnel, researchers and clinicians with EBV prevalence estimates and molecular epidemiology of EBV as a highly prevalent transfusion transmissible oncovirus.

Estimates of EBV infection are important to give researchers and clinicians correct data about how many people are affected, and to identify people at high risk groups such as pediatrics and immunocompromised patients. Also, it aids to ensure that safety health practices are followed to eliminate the EBV spread, especially in blood banks, and organ transplantation centers where EBV constitute a life threating risk to recipients. Moreover, EBV estimates in relation to age are highly needed to help in identifying the right age to introduce EBV vaccine in the future.

As an oncovirus with a complex cancer related etiology and high molecular variability, it is important to know what is the molecular epidemiology of EBV in this specific area and to identify the circulating EBV strains among the population. Finally, we believe that more studies should be conducted in this region to compare the EBV

strains between healthy carriers and patients with EBV associated diseases or malignancies.

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APPENDIX A. Qatar University IRB Approval

جامعة قطر
QATAR UNIVERSITY

Qatar University Institutional Review Board
QU-IRB

December 7, 2015

Dr. Gheyath K. Nasrallah
College of Arts and Sciences
Qatar University
Tel.: 4403-4817
Email: gheyath.nasrallah@qu.edu.qa

Dear Dr. Gheyath Nasrallah,

Sub.: Research Ethics Expedited Approval / UREP 18-001-3-001
Ref.: Project titled, "Detection and Phylogenetic Genotyping of Epstein Barr Virus among Blood Donors in Qatar"

We would like to inform you that your application along with the supporting documents provided for the above proposal, has been reviewed by the QU-IRB, and having met all the requirements, has been granted research ethics Expedited Approval for one year effective from December 7, 2015 till December 6, 2016.

Documents reviewed: Checklist, Application, Research Plan, QU-IBC Approval,

Please note that all approvals are valid for a period of one year and renewals should be sought one month prior to the expiry date to ensure timely processing and continuity. Moreover, any changes/modifications to the original submitted protocol should be reported to the committee to seek approval prior to continuation.

Your Research Ethics Approval No. is: **QU-IRB 518-EA/15**
Kindly refer to this number in all your future correspondence pertaining to this project.

Best wishes,

K. Alali

Dr. Khalid Al-Ali
Chairperson, QU-IRB

جامعة قطر
Institutional Review Board
(IRB)
Office Of Academic Research

APPENDIX B. Poster

Graduate Student, Biomedical and Health Sciences

Seroprevalence and Phylogenetic Genotyping of Epstein Barr Virus (EBV) among Blood Donors in Qatar

*Maria K. Smatti [1], Sarah A. Taleb [2], Asmaa Al Marwani [3], Raed AbuOdeh [4], Gheyath K. Nasrallah [1]**

[1]College of Health Sciences, [2] Biomedical Research Center, Qatar University; [3] Hamad Medical Corporation, [4]Universityof Sharjah

ABSTRACT

EBV is a lymphotropic herpesvirus and the causative agent of infectious mononucleosis. EBV is highly prevalent and has been linked to several malignancies. The virus is generally transferred by oral secretions and can be also transmitted through blood transfusions and organ transplantations. The goal of this study was to investigate the seroprevalence and the viremia rate of EBV among healthy blood donors, and to study the most prevalent EBV genotypes and subtypes in Qatar. For estimating EBV seroprevalence, serologic testing for the following EBV antibodies was performed: EBV viral capsid antigen (VCA) IgG &IgM, EBV nuclear antigen (EBNA) IgG & IgM, and early antigen (EA-D) IgG. To study the EBV viremia rate, DNA was extracted from plasma and subjected for EBV detection using RT-PCR. For genotyping, nested PCR with specific primers targeted EBNA genes were used. And for further subtyping, nested PCR with specific primers targeted LMP-1 genes were used. The PCR product of LMP-1 gene was then cloned, sequenced, and compared to previous sequences . Out of 673 analyzed samples (223 Qataris, 450 non-Qatari residents), 659 (97.9%) were seropositive for at least one of the anti-EBV antibodies. EBV viremia was detected in 354 (52.6%). (Genotyping of 51 randomly selected positive DNA samples showed that Genotype 1 was more prevalent, found in 36 (72.5%), while genotype 2 was found in 12 (23.5%) samples. Mixed infection of genotype 1&2 was found in 2 (3.9%). Sub genotyping by sequencing and phylogenetic analysis for 30 samples revealed that all tested clones (n=119) have the SEA-1 strain. 30-bp and 69 bp deletions in the LMP-1 were also found in 10% and 13.3% respectively. In conclusion, EBV is highly prevalent in Qatar, with predominance of genotype 1 and subtype 2. Deletions of LMP-1 also are detected in healthy carriers.

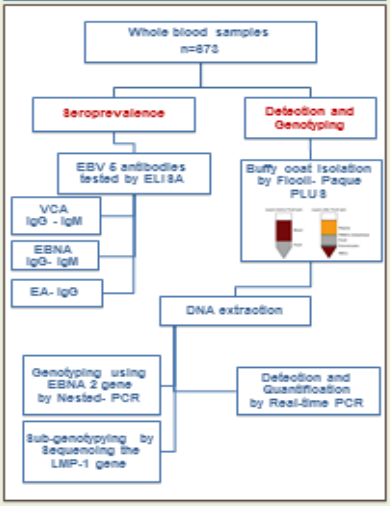
INTRODUCTION

Epstein Barr virus (EBV) was first discovered in cells isolated from African Burkitt's lymphoma, then it was realized that it is highly prevalent worldwide. It's estimated that more than 90% of world's populations is EBV-seropositive. There are two main EBV genotypes, Type 1 (A) and Type 2 (B) distinguished according to the variation in EBNA2 gene. LMP-1 gene is a highly variable, and it is used for further EBV subtyping. Deletions in LMP-1 gene have been reported in healthy individuals, as well as linked to the severity of EBV associated malignancies. EBV is generally transmitted by oral secretions. However, it can spread through blood transfusions and organ transplantation. Although leukoreduction can pointedly reduce the number of EBV genome, it doesn't eliminate the potential risk of EBV transmission. To our knowledge, this is the first study investigating the seroprevalence, the viremia rate, and the molecular epidemiology of EBV among blood donors in Qatar.

OBJECTIVES

(I) To investigate the seroprevalence and viremia rate of EBV among healthy blood donors.
(II) To Study the most prevalent EBV genotypes in Qatar
(III) To Study the molecular similarity of EBV isolates, using phylogenetic genotyping, compared to the reported EBV sequences in the rest of the world.

METHODS

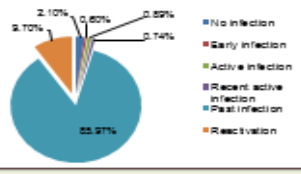


RESULTS

Table 1. Demographic characteristics of the studied subjects (N=673)

Category		No.(%)
Gender		
	Male	659 (97.9)
	Female	14 (2.1)
Nationality		
	Qatari	223 (33.1)
	Male	219 (98.2)
	Female	4 (1.8)
	Non-Qatari	450 (66.9)
	Male	440 (97.8)
	Female	10 (2.2)
Age		
	<20-30	176 (26.15)
	31-40	264 (39.2)
	41-50	174 (25.9)
	>50	59 (8.8)

Figure 1. Serologic diagnosis of the donors (N=673)



RESULTS

Figure 2. Seroprevalence in age groups (N=673)

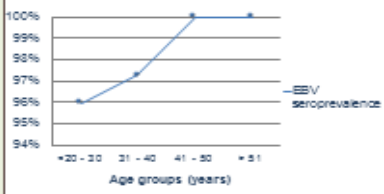


Table 2. EBV viremia rate

Total samples	673
Viremia rate	354, (52.6 %)
Viral load range	20 – 50,000 copies/ml

Figure 3. EBV genotypes (N=51)

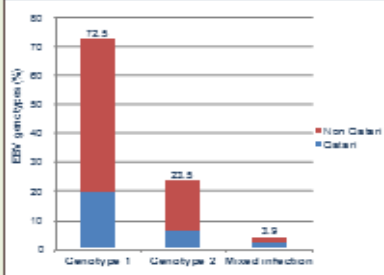


Table 3. Summary of Deleted and Non-Deleted Isolates (N=30)

EBV Genotype	Total no.	30 bp deletion (%)	69 bp deletion (%)	non deleted (%)
Type 1	25	3 (12)	4 (16)	18 (72)
Type 2	5	0 (0)	0 (0)	5 (100)
Total	30			

CONCLUSION

- EBV seroprevalence was 97.89%, and viremia rate was 52.6%, both significantly increase with age
- EBV Genotype 1 and Subtype SEA1 are the predominating (72.5%, 100%, respectively) in Qatar
- LMP-1 30 bp and 69 bp deletions were found in 10% and 13.3%, respectively.
- This study should provide the epidemiologists, blood banks' personnel, researchers and clinicians with EBV prevalence estimates and molecular epidemiology of EBV as a highly prevalent transfusion transmissible oncovirus.

APPENDIX C. Plagiarism Report