Sero-detection of Epstein Barr Virus antibodies among blood donors in Khartoum state

الكشف المصلي للاجسام المضادة لفيروس ابشتاين بار لدى متبرعين الدم بولاية الخرطوم

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By:
Meyassa Hassan Sayed Nasr
B.Sc Medical Laboratory Science • Sudan University of Science and Technology(2012)

Supervisor:
Prof. Yousif Fadlalla Hamed Alnil
Sudan University of Science and Technology

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بسم الله الرحمن الرحيم

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صدق الله العظيم
سورة الشرح
الآيات : 6-5
Dedication

To my parents

Who surrounded me with everlasting love and care

Who I live to make their dreams true.

To those who

Shared me happiness and sadness with great pleasure.
Acknowledgements

First of all a great thanks to ALMIGHTY ALLAH for giving me the power and willing to complete this study.

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Abstract

The main aim of this study was to determine the seroprevalence of Epstein Barr Virus (EBV) (VCA) IgG antibodies using enzyme linked immunosorbent assay (ELISA), among blood donors attending Blood Bank Department at National Public Health Laboratory in Khartoum State, from January to February 2015.

A total of 90 subjects were included, 75 blood donors and 15 controls with age ranging from 18-63 years. The results revealed that the prevalence of EBV (VCA) IgG in the total samples were 83(92.2%) and among blood donors was 69(92%) while 14(93.3%) in control subjects. Possible risk factors were examined in this study including age, sex, marital status, major blood groups and previous blood transfusion. All these showed no significant effect (P> 0.05) on EBV IgG antibodies among both blood donors and control group.

The results obtained showed that the prevalence of Epstein Barr Virus (VCA) IgG antibodies among both blood donors and control group increased with age, while it was 58(90.6%), 13(92.8%) in male, 11(100%), 1(100%) in female, 43(93.4%), 6(85%) in single, 26(89%), 8(100%) in married and 2(100%), 0(0%) in previously blood transfusion respectively.

The results showed that EBV is endemic in Sudan. Although primary EBV infection among normal immunocompetant individuals is not one of the clinically serious viral infections in Sudan, but the serious complications of diseases that occur in immunocompromised individuals (transplant recipients and AIDS) can not be neglected. This study recommends the screening of blood for EBV antibodies among blood and organ transplant donors.
ملخص الطروة

الهدف الرئيسي من هذه الدراسة هو تحديد مدى انتشار الأجسام المضادة من النوع IgG لفيروس إيبستاين بار باستخدام مقياس الامتصاص المناعي المرتبط بالإنزيم (الإيزا) عند متبرعين الدم في المعهد القومي الصحي بولاية National Public Health Laboratory الخرطوم في الفترة من يناير 2015 إلى فبراير من العام 2015.

شملت هذه الدراسة 90 شخسا منهم 75 متبرعا طوعيا و 15 مرادفا لهم أصحته طارئا (العينة الضابطة) ، في مدى عمري يتراوح من 18-63 سنة . حيث أظهرت أن معدل انتشار الأجسام المضادة لفيروس إيبستاين بار لدى إجمالي العينات (%) 92% و 69(%) عند المتبرعين بالدم و14(%) عند العينة الضابطة.

احتمت هذه الدراسة أيضا كل العوامل التي تزيد من معدل الإصابة بفيروس إيبستاين بار (مثل العمر ، الجنس ، الحالة الاجتماعية ، فصيلة الدم وكرار عملية نقل الدم) حيث تبين أنه ليس لها جدل احتماله (القيمة الإحصائية أكبر من 0.05) للأجسام المضادة من النوع IgG لفيروس إيبستاين بار لكل من المتبرعين بالدم والعينة الضابطة.

وكانت النتائج المتحصل عليها قد اظهرت ان معدل انتشار الأجسام المضادة من النوع IgG لفيروس إيبستاين بار لكل من المتبرعين بالدم والعينة الضابطة يترافق مع التقدم في العمر بينما كانت (90.6%) (13%) عند الرجال ، (100%) عند النساء ، (58%) عند غير المتزوجين ، (26%) (8%) عند المتزوجين.

2(%) 0% (0%) لمن تكرر لهم نقل دم على التوالي.

أظهرت النتائج في هذه الدراسة ان فيروس إيبستاين بار مستوطن في السودان ، على الرغم من ذلك تعتبر الإصابه الأولية لدى الأشخاص طبقي المناعة غير مهمة سريريا ولكن نظرا للمضاعفات والإمراض الخطيرة التي لا يمكن تجاهلها لدى الأشخاص واهني المناعة مثل مرضى زراعة الأعضاء والمصابين بالإيدز نوصى بشدة ضرورة إجراء المسح المفصلي للأجسام المضادة لفيروس إيبستاين بار لكل من المتبرعين بالدم والأعضاء.

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CHAPTER ONE
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1. Introduction

1.1 Introduction:

Herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), varicella zoster virus (VZV), Epstein-Barr virus (EBV), and cytomegalovirus (CMV) are human herpesviruses (HHV) that are prevalent worldwide, their infections can lead to a variety of clinical conditions that range in severity from cold sores and genital ulcers (HSV), and chickenpox (VZV) to potentially sight-threatening (e.g. CMV uveitis and HSV keratitis) or even life-threatening diseases such as HSV encephalitis and EBV-associated malignancies (Schaftenaar et al; 2014).

Primary HHV infections, commonly acquired at young age, lead to a lifelong latent infection with intermittent reactivation resulting in periodic asymptomatic or recrudescent disease. The host immune system is pivotal to resolve lytic infections and to inhibit HHV reactivation from latency (Schaftenaar et al; 2014).

Epstein-Barr virus (EBV) belongs to the family Herpesviridae, subfamily Gammaherpesvirinae and Genus Lymphocryptovirus, which represent the most important human pathogens (Saravani et al; 2014).

The virion is Enveloped, icosahedral nucleocapsid symmetry, spherical to pleomorphic particle, 95-105 nm in diameter, and has linear, double-stranded DNA genome about 184 kbp in length. Between the capsid and the envelope is an amorphous layer of proteins termed the tegument (Sathiyaamoorthy et al; 2014).

Primary Epstein-Barr virus (EBV) infection usually occurs within the first years of life. At an early age, the infection is usually asymptomatic, whereas, during adolescence and adulthood, it can present as acute
infectious mononucleosis (IM). The infection is extremely common, and > 90% of adults are seropositive for EBV (Helminen et al; 2015). The infection spread through salivary contact, and the mucosal epithelium of the oropharynx is considered to be the first site of infection and replication. From the oropharynx, the virus is transmitted to locally infiltrating B cells, where it persists for a person’s life (Helminen et al; 2015).

It is an oncogenic virus associated with a wide array of human tumors including epithelial cell tumors such as nasopharyngeal and gastric carcinomas, and lymphoid malignancies like Hodgkin and Burkitt lymphoma. Subsequent reactivations of the virus are asymptomatic and managed effectively by the immune system in healthy adults. However, immunocompromised patients suffer from severe opportunistic disorders, such as post-transplant lymphoproliferative disease (PTLD), oral hairy leukoplakia and HIV/AIDS related malignancies ( Sathiyamoorthy et al; 2014).
1.2 Rationale:
Epstein-Barr virus (EBV) is an important human pathogen with a worldwide distribution and one of the most common viral infection in humans (Crowcroft et al; 1998).
Epstein-Barr virus is one of the transfusion transmissible viruses with others like hepatitis B virus (HBV), hepatitis C virus (HCV), human immunodeficiency virus (HIV), human T cell leukaemia virus I (HTLV1) and cytomegalovirus (CMV) (Iheanacho et al; 2014).
Epstein - Barr virus (EBV) - related post- transplant lymphoproliferative disease (PTLD) is one of the most serious complications associated with solid organ and hematopoietic stem cell transplantation. PTLD is most frequently seen with primary EBV infection post-transplant, a common scenario for pediatric solid organ recipients. Risk factors for infection or reactivation of EBV following solid organ transplant are stronger immunosuppressive therapy regimens, and being seronegative for receptor. Mortality is high, and most frequent in patients who develop PTLD in the first six months post-transplant (Green; 2001).
Latent EBV infection of B lymphocytes in the blood of healthy donors affords another potential route of transmission; infection has been documented after infusion of a large volume of fresh blood (Macsween and Crawford; 2003).
EBV is a neglected virus but can cause serious problems that cannot be neglected and since no previous studies was done in Sudan to determine the seroprevalence of Epstein Barr Virus antibodies in blood donors.
1.3 Objectives:

1.3.1 General objective:
To investigate the seroprevalence of Epstein Barr Virus antibodies among blood donors attending National Public Health Laboratory in Khartoum State.

1.3.2 Specific objectives:
- To detect the specific Epstein Barr Virus IgG(VCA) in serum obtained from donors using enzyme linked immunosorbent assay (ELISA).
- To find out the major risk factors associated with EBV infection among blood donors, by using interviewing questionnaire (sex, age, marital status, number of blood transfusion and a major blood groups like ABO and Rhesus factor).
CHAPTER TWO
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2. Literature review

2.1 Historical background:
Epstein Barr Virus (EBV) was discovered as a result of pioneering work in the 1950s, by Denis Burkitt. Burkitt identified a previously unrecognized form of cancer which affected the jaws of young African children, and he made the crucial insight that the distribution of this common tumor (now known as Burkitt’s lymphoma) appeared to be influenced by climatic factors -- notably temperature elevation. Burkitt theorized that the tumor might be due to a mosquito-born virus, or arbovirus (Tortora et al; 2004).

This discovery led Michael Epstein, Yvonne Barr and Burt Achong to examine freshly excised tumor biopsies for the presence of a virus. In 1964, using electron microscopy, they found herpesvirus-like particles in a small number of the biopsied cells, and they subsequently established that this was in fact a new virus. Epstein-Barr virus was thus identified as the first candidate human tumor virus (Bauman; 2011).

A fourth human herpes virus is Epstein-Barr virus (EBV) which in the family Herpesviridae , subfamily Gammaherpesvirinae and Genus Lymphocryptovirus. The official name for the species is human herpesvirus 4 , or HHV-4 (Saravani et al; 2014).

2.2 Structure of virus:
Figure 2.1 illustrates the general structure of the virus ( see in appendix-2). Like all herpesviruses, the Epstein-Barr virus is relatively large and complex. The virus's structure consists of an envelope, envelope protein (spikes), viral genome (core), anucleocapsid and viral tegument. All these structures aid in making the virus successful in the infection process
and as a means of avoiding detection from our body's immune system (Ebell; 2004).

2.2.1 Envelope protein (Spikes):
The "spikes" are glycoproteins that coat the outside surface of the virus envelope. These spikes are a means of attachment for the virus when it encounters potential host cells. With these proteins, the virus can latch on to the host cell surface and begin the replication cycle. These proteins are also a means of finding host cells, as their proteins will bind only with those specialized receptor cells on the host (Ebell; 2004).

2.2.2 Envelope:
The envelope is a protective outer membrane that surrounds the virus, it is covered by the glycoproteins mentioned above. The virus acquires these proteins and envelope when it leaves a host cell. The virus buds out of the host cell and takes the membrane along with its receptor proteins. The viral envelope is not very sturdy and can be easily damaged. A damaged envelope means that the virus is not able to infect host cells and therefore is not able to cause pathology in people. While the envelope does protect the virus and hold it together structurally, it does have its downsides. The membrane is very sensitive to drying out, acids, detergents, and many other organic solvents. This occurs because of the membrane being made of lipids (Ebell; 2004).

2.2.3 Viral genome (Core):
The core holds the double-stranded DNA and encloses inside all of the other structures. The core has a toroidal shape, which means that it resembles a donut. The DNA is wound around special proteins in the middle of the core. The core measures about 75 nanometeres in diameter (Ebell; 2004).
2.2.4 Nucleocapsid :
The capsid surrounds the core and protects the genetic information (double-stranded DNA). The capsid itself has a nucleocapsid with an icosahedral shape, which means it has twenty sides. The capsid measures about 95-105 nanometers in diameter (Ebell; 2004).

2.2.5 Viral tegument :
The tegument is the space between the capsid and envelope. This space is filled with proteins, and handles proteins and enzymes needed for replication and prepares them to do so (Ebell; 2004).

2.3 Virus replication :
The tissue tropism of EBV in vivo is mainly restricted to B lymphocytes and epithelial cells. Virus infection in B lymphocytes is mainly latent, whereas in epithelial cells, it is lytic, i.e., productive. EBV infection in B lymphocytes and epithelial cells is initiated by attachment of virions to the cell surface (Tugizov et al; 2013).
Viral membrane fusion is a requisite step for infection for all lipid bilayer encased viruses, such as the herpesviruses, and requires one or several virus-encoded glycoproteins that orchestrate the merging of viral and host membranes in a step-wise manner. This overall process leads to the release of viral capsid into the host cytoplasm initiating infection (Sathiyamoorthy et al; 2014).
The entry of EBV into B cells is complex and involves at least five different glycoproteins (EBV gp350/220, gH, gL, gp42 and gB). Of these five proteins, four (gH, gL, gB and gp42) are indispensable for membrane fusion with B cells and three (gH, gL and gB) are required for fusion with epithelial cells. gH, gL and gB form the core fusion machinery common to all herpesviruses (Sathiyamoorthy et al; 2014). During B cell entry, EBV gp350/220 binds to complement receptor 2 (CR2/CD21) concentrating virus to the B cell surface, but this interaction
does not activate membrane fusion or virus entry (Sathiyamoorthy et al; 2014).

The gp42 protein forms stable, high affinity complexes with the gHgL complex, and also binds to human leukocyte antigen (HLA) class II which acts as the triggering receptor for EBV entry into B cells (Sathiyamoorthy et al; 2014).

2.4 Transmission of infection:

EBV replicates primarily in B-lymphocytes but also may replicate in the epithelial cells of the pharynx and parotid duct. The infection is spread primarily by saliva, and the incubation period is four to eight weeks (Ebell; 2004).

However, there are single reports of EBV detection in male and female genital secretions, suggesting the possibility of sexual transmission. A recent seroepidemiological study on university students lends support to this possibility by showing strong correlations of both EBV seropositivity and history of infectious mononucleosis (IM) with sexual intercourse and increasing numbers of sexual partners (Macsween and Crawford; 2003). However, these data are not conclusive because they do not differentiate between direct transmission in genital secretions and spread by practices associated with sexual intercourse such as kissing (Macsween and Crawford; 2003).

Latent EBV infection of B lymphocytes in the blood of healthy donors affords another potential route of transmission. Infection has been documented after infusion of a large volume of fresh blood. Transmission from a transplanted organ can also occur with subsequent infection of a previously seronegative recipient and is a risk factor for post-transplant lymphoproliferative disease (PTLD) (Macsween and Crawford; 2003).
2.5 Epidemiology:
More than 90% of adults worldwide have been infected with EBV and carry the virus as a life-long persistent infection with latent infection of B lymphocytes and virus production into saliva. In most cases primary infection occurs subclinically during childhood, often by spread between family members via salivary contact (Macsween and Crawford; 2003). Epidemiological studies showed that primary infection occurs early in non-industrialised countries and in low socioeconomic groups, whereas in affluent societies seroconversion may be delayed until adolescence, when infectious mononucleosis (IM) develops (Macsween and Crawford; 2003).

It is commonly assumed that those who remain uninfected throughout childhood generally become infected as adolescents through kissing, and consequently IM is often called the kissing disease (Macsween and Crawford; 2003).

The seropositivity of EBV IgG antibody using ELISA technique was not performed in Sudan among blood donors, but there are studies were performed in other countries. Studies were performed by Sousa et al (2011) in Portugal (95%), Macsween and Crawford (2003) in UK (>90%), Helminen et al (2001) in Finland (>90%), Saravani et al (2014) in Iran (>90%), Hurme and Helminen (1998) in Finland (95%) and by Lazda (2006) in USA which was (94%).

2.6 Pathology and pathogenecity:
EBV associated diseases often arise from a failure of the host immune response to control the proliferation of latently infected cells. This is the opposite of what happens in other herpesviruses, where the problem is a failure to respond to lytic infection, and this correlates with the fact that
latent infections by EBV predominate in vivo, with lytic-phase infection occurring in very few cells (Sugden; 1994).

EBV infection is usually asymptomatic in childhood, and about 90% of adults are positive for the virus. EBV is spread by saliva and the virus initially infects oropharyngeal epithelial cells, where it replicates efficiently. The virus then infects B cells, as they pass through the oropharynx and this results in the establishment of latent viral infection in B cells and persists lifelong. EBV infection of B cells is associated with the rapid proliferation and expansion of EBV+ B cells during primary viral infection (Sugden; 1994).

Normally, this EBV-driven proliferation of B cells is brought under control by cytotoxic T cells (CTLs). This commonly results in an infectious mononucleosis (IM), particularly in young adults. However, in certain individuals, the initial EBV-driven B cell proliferation is not adequately contained, and this may result in fatal IM, which occurs particularly in males with X-linked lymphoproliferative disorder (XLP) (Sugden; 1994).

During the persistent stage of EBV infection (i.e., following the resolution of the acute infection), the virus primarily infects long-lived memory B cells in the periphery. This is thought to allow the growth-promoting genes of the virus to be switched off to create a site of persistent infection in vivo, without causing disease or providing antigenic targets for the immunosurveillance (Sugden; 1994).

2.7 Clinical significance:
As stated earlier, primary infection in infancy or childhood is usually asymptomatic, but as high as fifty percent of those infected later in life develop the disease infectious mononucleosis. Although B cells are the primary target of infection due to infection due to the presence of the EBV receptor molecule, EBV has more recently been found to be
associated with a small number of Tcell malignancies as well (Strohl et al; 2001).

In patients who are immunodeficient or immunosuppressed, the lack of cell mediated immune control increases the likelihood of lymphoproliferative disorder of various kinds. Throughout life, healthy EBV carriers continue to have episodes of a symptomatic virus shedding (Strohl et al; 2001).

The source of this virus is presumably productively infected oropharyngeal cells that acquire the virus from latently infected B cells in which the lytic cycle has been activated (Strohl et al; 2001).

2.7.1 Infectious mononucleosis:

The manifestations and severity of primary EBV infection vary greatly, but the typical IM syndrome appears after an incubation period of four to seven weeks, and includes pharyngitis, lymphadenopathy, increased levels of liver enzymes in the blood, fever. Headache and malaise often precede and accompany the disease, which may last several weeks. Complete recovery may take much longer (Strohl et al; 2001).

2.7.2 EBV and malignancies:

Since the initial discovery of EBV in association with Burkitts lymphoma (BL), it has been shown to be associated with a number of other human neoplastic diseases (Strohl et al; 2001).

2.7.2.1 Burkitts lymphoma (BL):

BL was first described in 1985 as a rather unique malignancy of the jaw, found at unusually high frequency in children in regions of equatorial Africa. Distribution of Burkitts Lymphoma (see appendix-2). Malaria and HIV infection are known risk factors for development of BL (Strohl et al; 2001).

Burkitts lymphoma is thought to result from an early EBV infection that produces a large pool of infected B lymphocytes. Such stimuli can lead
to chromosomal translocation, which are pathognomonic for this lymphoma (Ahmad et al; 2010).

2.7.2.2 Nasopharyngeal Carcinoma (NPC):

It is one of the most common cancers in Southeast Asia, North Africa, and among the Eskimo population, but less common elsewhere (Strohl et al; 2001).

NPC differs from BL in that there is no characteristic chromosomal alteration, and the cells involved are epithelial in origin. The role of EBV is indicated by the fact that all cells of the tumor contain cytoplasmic viral DNA molecules (Strohl et al; 2001).

2.7.2.3 EBV infections in immunocompromised and immunosuppressed patients:

In BL and NPC, EBV infection appears to be only one step in a multi-step, disease-causing process, and its specific role is still not well defined (Strohl et al; 2001).

In contrast, EBV alone appears to be sufficient for induction of B-cell lymphomas in immunocompromised patients, such as transplant recipients and individuals with AIDS, who cannot control the cell multiplication induced by the early protein (Strohl et al; 2001).

For example, many AIDS patients develop a B cell malignancy of some type: BL of the sporadic type occurs with high frequency in the earlier stages of AIDS progression, where as non-BL-type lymphoplastic lymphomas are more characteristic in late stage AIDS patients (Strohl et al; 2001).

All of the HIV-associated BL cases contain the EBV genome. And AIDS patients infected with EBV may also exhibit nonmalignant, white-grey lesions on the tongue "hairy leukoplakia" (Strohl et al; 2001).
2.7.2.4 Lymphoproliferative syndrome:

Patients with primary or secondary immunodeficiency are susceptible to EBV-induced Lymphoproliferative disease. For example, the incidence of these lymphomas is 1% to 2% after renal transplantations and 5% to 9% after heart-lung transplantations (Ahmad et al; 2010). The risk is greatest in patients experiencing primary EBV infection rather than reactivation. Most characteristic is persistent fever lymphadenopathy and hepatosplenomegaly (Ahmad et al; 2010).

2.7.3 EBV and autoimmune diseases:

Epidemiological data suggest that EBV is associated with several autoimmune diseases, such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and multiple sclerosis (MS). However, it is not clear whether EBV plays a role in the pathogenesis of these diseases, and if so, by which mechanisms the virus may contribute. (Lossius et al; 2012).

A possible association between EBV seropositivity and autoimmune diseases was first observed by coincidence in 1968 in a Brazilian population. Since then, a multitude of studies have explored humoral immunity against EBV in SLE, RA and MS. North Americans of different ethnicities with SLE had an increased seroprevalence of EBV and studies in other populations have shown an increased frequency of antibodies against EBV early antigens (Lossius et al; 2012). In one study, 99% of young SLE patients were seropositive for EBV compared to 70% of age-matched controls. Almost all adult MS patients are seropositive for EBV, compared to 90% of healthy adults. As for SLE, the differences in seroprevalence are more pronounced in lower age groups, where the general seroprevalence is lower. Further, it has been demonstrated that MS risk is very low in individuals not infected with EBV, but increases sharply after EBV infection (Lossius et al; 2012).
In a recent meta-analysis, previous EBV infection was actually found to be present in 100% of MS patients in studies using two independent methods of antibody detection. The authors claimed that findings of MS patients without earlier EBV infection could be due to low sensitivity in the assays used for detection of antibodies (Lossius et al; 2012).

In SLE and MS, titers of antibodies against EBV antigens are elevated compared to healthy controls and for both diseases, this elevation seems to predate the first symptoms (Lossius et al; 2012).

2.7.4 X-linked lymphoproliferative syndrome:
X-linked lymphoproliferative syndrome (XLPS or XLP) is a rare, familial, fatal form of IM that has been recognized for almost 30 years. Typically XLP affects young males who are clinically well before primary EBV infection, but when infected most rapidly succumb to fulminant IM (Macsween and Crawford; 2003).

2.8 Host Response to EBV Infection:
Antibodies to early antigens diffuse (EA-D), methanol-resistant and restricted (EA-R), methanol-sensitive as well as EBNA-2 rise and fall with convalescence, while antibodies to EBNA-1 develop only after convalescence and persist at a low titer for life. Antibodies to the gp350 envelope protein, known as membrane antigen (MA), rise slowly during acute infection and persist after convalescence. The asymptomatic post-convalescent carrier state is characterized by persistent IgG antibody to VCA, MA, and EBNA-1 (Iwatsuki et al; 2004).

Virus reactivation occurring in immunocompromised virus carriers is marked by rising titers of IgG anti-VCA and anti-EA antibody, and accompanied by a rise in viral load in blood as detected by real-time quantitative EBV PCR. In addition to virus-specific antibodies, patients with EBV-associated infectious mononucleosis rapidly and transiently develop heterophile antibodies, low titer IgM antibodies of unknown
primary specificity that agglutinate heterologous (sheep, horse, cow) red blood cells (Iwatsuki et al; 2004). Heterophile antibodies are both sensitive and specific for EBV-associated infectious mononucleosis since they are not usually seen in infectious mononucleosis syndromes associated with other infections. The EBV-specific T cell response in acute primary infection (infectious mononucleosis) is dominated by CD8+ CTL with lytic antigen specificity, with a proportional increase in latent antigen specificity following recovery (Iwatsuki et al; 2004).

Lytic proteins targeted by EBV-specific CD8+ T cells are most often immediate early or early proteins rather than late proteins. The CD8+ T-cell response to latent antigens is largely targeted to EBNA-3 proteins (3A, 3B, 3C). The change in CD8+ T-cell antigen specificity likely reflects the biology of infection—acute primary infection initiated by a burst of lytic replication followed by immune suppression of lytic replication and establishment of a persistent pool of latent-infected B cells. In contrast to the CD8+ T-cell response, less is known about the CD4+ EBV-specific T-cell response during infectious mononucleosis (Iwatsuki et al; 2004).

2.9 Laboratory identification:

The most commonly used diagnostic criterion is the presence of 50% lymphocytes with at least 10% atypical lymphocytes (large, irregular nuclei), in the blood smear of a patient with IM. The atypical lymphocytes resembled monocytes when they were first discovered, thus the term "mononucleosis" was coined. Infectious mononucleosis in peripheral smear, showing reactive lymphocytes (Murray et al; 2007) (see in appendix -2). Detection of EBV DNA or RNA by hybridization, or of virus antigens using immunohistochemical techniques, can be done with cell
homogenates or by *in situ* methods for visualization of individual infected cells (Strohl *et al*; 2001).

The "classic" test for infectious mononucleosis, the Paul-Bunnell-Davidsohn test, is based upon the fact that polyclonal stimulation of B cells by EBV infection results in a nonspecific elevation of hetrophile antibodies that specifically agglutinate horse and sheep red blood cells. These hetrophile antibodies are diagnostic for EBV-related IM, although they are not present in all cases of EBV IM (Strohl *et al*; 2001).

**2.10 Treatment:**

Treatment of infectious mononucleosis is largely supportive. More than 95% of patients recover uneventfully. In a small percentage of patients, splenic rupture may occur; restriction of contact sports or heavy lifting during the acute illness is recommended (Ahmad *et al*; 2010).

The DNA polymerase enzyme of EBV has been shown to be sensitive to a cyclovir, and a cyclovir can decrease the amount of replication of EBV in tissue culture and in vivo (Ahmad *et al*; 2010). Despite this anti-viral activity, systemic a cyclovir makes little or no impact on the clinical illness. Laryngeal obstruction should be treated with corticosteroids. Hairy leukoplakia in AIDS patients dose respond to acyclovir treatment (Ahmad *et al*; 2010).

**2.11 Prevention:**

The occurrence of Burkitt lymphoma and nasopharygeal carcinoma in restricted geographic areas offers the possibility of prevention by immunization with virus specific antigens (s) (Ahmad *et al*; 2010).
CHAPTER THREE
CHAPTER THREE

3. Materials and methods

3.1 Study design :
A prospective cross sectional study.

3.2 Study area and duration :
This study was conducted in Blood Bank Department at National Public Health Laboratory and the experimental work was done in Research laboratory in Sudan University of Science and Technology, from January to February 2015.

3.3 Study population :
All blood donors attending the Blood Bank for donation of blood during the study period were considered eligible to be included in the study irrespective of age, sex and residence. Control subjects were randomly selected from apparently healthy individuals.

3.4 Sample size :
A total of 90 subjects were included, 75 blood donors and 15 controls to study the prevalence of EBV.

3.5 Sampling technique :
The study is based on non-probability convenience sampling technique. Samples were taken from blood donors and control during the period of donation after their agreement to participate.

3.6 Method of data collection :
Data were collected according to personal structured interview (see questionnaire in appendix-1).

3.7 Ethical consideration :
The blood donors and control subjects were informed for the purpose of the study and its objectives, before taking their permission, with protections of their privacy. Permission to carry out the study was taken.
from the college of graduate studies, Sudan University of Science and Technology.

3.8 Specimen collection:
Aliquots of five ml of venous blood were collected by venous puncture after sterilizing the site of collection. The collected blood was drawn into plain containers, allowed to clot and then centrifuged at 3000 rpm for 5 minutes. Sera were separated into new sterile ependroff tubes preserved at 2°C – 8°C until used. Hemolytic or lipemic or icteric specimens were excluded.

3.9 Laboratory work:
The preserved sera for both blood donors and control group were then tested for Epstein Barr Virus IgG(VCA) antibodies using ELISA. Figure 3.1 show microtiter ELISA plate (see appendix-2).

3.10 Principle:
The test kit contains micro titer strips with 8 break-off reagent wells coated with EBV-CA. In the first reaction step, diluted samples are incubated in the wells. In the case of positive samples, specific IgG antibodies will bind to the antigens. To detect the bound antibodies, a second incubation is carried out using an enzyme labelled anti-human IgG (enzyme conjugate) catalysing a colour reaction. A third incubation is carried out using chromogen/substrate solution. The reaction is then stopped by adding acid. The result of colours are read by micro ELISA reader and then the reading of samples is compared with the controls and calibrators (EUROIMMUM, Germany).

3.11 Procedure:
According to manufacturer guidelines (EUROIMMUM, Germany) the following steps were followed (see appendix-3):
All reagents were brought to room temperature approximately 30 minutes before used. Diluted washing buffer was prepared by adding 40 ml to 360 ml of distilled water in flask. Samples were diluted by adding 10µl to 1 ml of samples diluent. Aliquots of 100 µl of calibrators, controls and diluted sera were dispensed into appropriate wells, incubated for 30 min at room temperature (18°C to 25°C) and then washed three times using 300 µl diluted washing buffer.

100 µl of enzyme conjugate (Peroxidase–labeled anti-human IgG) were dispensed into all wells, incubated for 30 min at room temperature (18°C to 25°C) and then washed three times using 300 µl diluted washing buffer.

100µl of chromogen/substrate solution (TMB/H₂O₂) were dispensed into all wells, incubated for 15 min at room temperature.

100µl of stop solution (0.5 M sulphuric acid) were added to the wells containing chromogen/substrate solution.

The colour intensity was read at a wavelength of 450 nm within 30 minutes of adding the stop solution.

3.12 Calculation and interpretation of the result:

The test was considered valid if the range of

Calibrator 1 O.D. > 0.700
Calibrator 2 O.D. > 0.140
Positive Control Ratio 1.9 - 4.9
Negative Control Ratio 0 – 0.7

Results were evaluated semiquantitatively by calculating a ratio of the extinction value of the control or patients sample over the extinction
value of the calibrator 2. The calculation of the ratio were done according the following formula:

\[
\frac{\text{Extinction value of the control or patients sample}}{\text{Extinction value of the calibrator 2}} = \text{Ratio}
\]

EUROIMMUN recommends interpreting results as follows:

- Ratio < 0.8 : negative
- Ratio ≥ 0.8 to < 1.1 : borderline
- Ratio ≥ 1.1 : positive

3.13 Data analysis

Data were analyzed by using SPSS software program version 16.0. Data were presented in form of tables. Significance of differences was determined using Chi-square test and statistical significance was set at p-value < 0.05.
CHAPTER FOUR
4. Results

4.1 Detection of anti-EBV IgG among the blood donors and control group

The results in table 4.1 demonstrated that 92% (69 out of 75) were anti-EBV IgG positive and only 8% (6) were EBV IgG negative, while the control subjects 93.3% (14 out of 15) were anti-EBV IgG positive and only 6.7% (1) was EBV IgG negative (Table 4.1).

Table 4.1 The seroprevalence of anti-EBV IgG positive cases among the blood donors and control group

<table>
<thead>
<tr>
<th>Study group</th>
<th>Anti-EBV IgG positive *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. Examined</td>
</tr>
<tr>
<td>Blood donors</td>
<td>75</td>
</tr>
<tr>
<td>Control group</td>
<td>15</td>
</tr>
<tr>
<td>Total</td>
<td>90</td>
</tr>
</tbody>
</table>

*(P > 0.05)

4.2 The effect of sex and marital status on EBV IgG prevalence

The results in table 4.2 revealed that there were no significant difference (P > 0.05) between males or females and between single or married blood donors and control group on EBV IgG prevalence (Table 4.2).
Table 4.2 The effect of sex and marital status on EBV IgG prevalence

<table>
<thead>
<tr>
<th>Target group</th>
<th>Anti-EBV IgG positive *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blood donors</td>
</tr>
<tr>
<td></td>
<td>No. Examined</td>
</tr>
<tr>
<td>Males</td>
<td>64</td>
</tr>
<tr>
<td>Females</td>
<td>11</td>
</tr>
<tr>
<td>Single</td>
<td>46</td>
</tr>
<tr>
<td>Married</td>
<td>29</td>
</tr>
</tbody>
</table>

* (P > 0.05)

4.3 The effect of age on EBV IgG prevalence

The results in table 4.2 showed that the age of blood donors and control group had no significance effect (P > 0.05) on EBV IgG prevalence (in table 4.2).

Table 4.3 The effect of age on EBV IgG prevalence

<table>
<thead>
<tr>
<th>Age group (years)</th>
<th>Anti-EBV IgG positive *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blood donors</td>
</tr>
<tr>
<td></td>
<td>No. Examined</td>
</tr>
<tr>
<td>18-25</td>
<td>40</td>
</tr>
<tr>
<td>26-33</td>
<td>21</td>
</tr>
<tr>
<td>34-41</td>
<td>10</td>
</tr>
<tr>
<td>42-49</td>
<td>2</td>
</tr>
<tr>
<td>50-57</td>
<td>2</td>
</tr>
<tr>
<td>58-65</td>
<td>0</td>
</tr>
</tbody>
</table>

*(P > 0.05)
4.4 The effect of previous blood transfusion on EBV IgG prevalence

Previous blood transfusion showed no significant difference ( $P > 0.05$ ) on blood donors and control group on EBV IgG prevalence (Table 4.4). Table 4.4 effect of previous blood transfusion on EBV IgG prevalence

<table>
<thead>
<tr>
<th>Blood transfusion</th>
<th>Anti-EBV IgG positive *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blood donors</td>
</tr>
<tr>
<td></td>
<td>No. Examined</td>
</tr>
<tr>
<td>Yes</td>
<td>2</td>
</tr>
<tr>
<td>No</td>
<td>73</td>
</tr>
</tbody>
</table>

*( $P > 0.05$ )

4.5 The effect of major blood groups ( ABO and Rhesus ) on EBV IgG prevalence

The results explained that the major blood groups of both blood donors and control group had no significance effect ( $P > 0.05$ ) on EBV IgG prevalence, and the EBV IgG present irrespective of blood group (Table 4.5).
Table 4.5 The effect of major blood groups (ABO and Rhesus) on EBV IgG prevalence

<table>
<thead>
<tr>
<th>Target Group</th>
<th>Anti-EBV IgG positive *</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blood donors</td>
<td>Control group</td>
</tr>
<tr>
<td></td>
<td>No. Examined</td>
<td>No. Positive</td>
</tr>
<tr>
<td>A</td>
<td>29</td>
<td>27</td>
</tr>
<tr>
<td>B</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>AB</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>O</td>
<td>36</td>
<td>32</td>
</tr>
<tr>
<td>Total</td>
<td>75</td>
<td>69</td>
</tr>
</tbody>
</table>

*(P > 0.05)

<table>
<thead>
<tr>
<th>Target Group</th>
<th>Anti-EBV IgG positive *</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blood donors</td>
<td>Control group</td>
</tr>
<tr>
<td></td>
<td>No. Examined</td>
<td>No. Positive</td>
</tr>
<tr>
<td>Rhesus Positive</td>
<td>73</td>
<td>67</td>
</tr>
<tr>
<td>Rhesus negative</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>75</td>
<td>69</td>
</tr>
</tbody>
</table>

*(P > 0.05)
CHAPTER FIVE
CHAPTER FIVE

5. Discussion

5.1 Discussion:
Primary EBV infection triggers a humoral immune response, an innate NK cell response, and a CTL response. Shortly after infection, rapid rise and fall of IgM anti-VCA (virus capsid antigen) antibody is soon followed by IgG anti-VCA antibody that persists at a low titer for life. In this study, the enzyme-linked immunosorbent assay (ELISA) was used for the detection of EBV IgG antibodies in both voluntary blood donors and control subjects.
Seroprevalence of EBV IgG (VCA) was found to be almost equal in both target groups (92% blood donors, 93.3% control group). Result is in agreement with those previously reported among blood donors by Sousa et al (2011) in Portugal (95%), Macsween and Crawford (2003) in UK (>90%), Helminen et al (2001) in Finland (>90%), Saravani et al (2014) in Iran (>90%), Hurme and Helminen (1998) in Finland (95%) and by Lazda (2006) in USA which was (94%).
The results obtained in this study demonstrated that EBV IgG develops with progression of age and this agree with Lazda (2006). The study also showed that there was no significant difference (P > 0.05) between males or females, between single or married blood donors and control group on EBV IgG prevalence.
Although previous repeated blood transfusion were associated with increased risk of EBV transmission, this study did not show significant effect of this risk factor on EBV infection among both target populations. In fact only 2 individual from blood donors received blood transfusions, a very low number that can not reflect the real effect of this important predisposing factor, since there is no previous study in the effect of the major blood groups on EBV infection and transmission. This study revealed no effect of the ABO and Rhesus blood groups of the two target
groups on the EBV prevalence, indicating no direct association of this factor to EBV infection and transmission.
5.2 Conclusion:
Based on the result of this study it concluded that EBV is not only common, but highly endemic in Sudan. Although not significant, but some predisposing factors showed potential effects on EBV infection.

5.3 Recommendations:
According to this study EBV infection was found to be widely distributed among Sudanese people, especially among blood donors, hence the following suggestions are strongly recommended:

- All blood and organ donors should be routinely screened for EBV infection before donation, especially for immunocompromised patients.
- Further indepth studies, such as virus isolation and molecular technique are required to be done on EBV prevalence.
References
References


Appendices
Appendix-1
Seroprevalence of Epstein Barr Virus IgG(VCA) among Blood Donors:

1. Date …………….
2. Name …………
3. Sample No……….
4. Gender
   Male ☐        Female ☐
5. Age …………..
6. Marital status
   Single ☐       Married ☐
7. Blood group …………..
8. Rhesus …………..
9. Blood transfusion
   Yes ☐          No ☐

Result

Epstein Barr Virus IgG(VCA) …………..
Appendix-2
Figure 2.1 illustrates the general structure of the virus

Figure 2.2 Distribution of Burkitt’s Lymphoma

*Distribution of Burkitt’s Lymphoma (Association with areas of low elevation)*

Figure 2.3 Infectious mononucleosis in peripheral smear, showing reactive lymphocytes.

Figure 3.1 show microtiter ELISA plate.
Appendix-3
## Qualitätskontrollzertifikat

**Quality Control Certificate**

<table>
<thead>
<tr>
<th>Produkt: Anti-EBV-CA ELISA (IgG)</th>
<th>Best.-Nr.: El 2791-9601 G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ch.-B.: E141110BT</td>
<td>Verw. bis: 09-Nov-2015</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Referenzwert</th>
<th>Valider Bereich</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reference value</td>
<td>Valid range</td>
</tr>
<tr>
<td>Kalibrator 1</td>
<td>200 RU/ml</td>
<td>1,755 (O.D.) &gt; 0,700 (O.D.)</td>
</tr>
<tr>
<td>Kalibrator 2</td>
<td>20 RU/ml</td>
<td>0,308 (O.D.) &gt; 0,140 (O.D.)</td>
</tr>
<tr>
<td>Kalibrator 3</td>
<td>2 RU/ml</td>
<td>0,085 (O.D.)</td>
</tr>
</tbody>
</table>

| Pos. Kontrolle 1  | quantitativ | 111 RU/ml | 78 - 144 RU/ml |
| Pos. Kontrolle 1  | semiquantitativ | 3,4 Ratio | 1,9 - 4,9 Ratio |
| Neg. Kontrolle    | quantitativ | 2 RU/ml | 0 - 15 RU/ml |
| Neg. Kontrolle    | semiquantitativ | 0,1 Ratio | 0 - 0,7 Ratio |

O.D. Kalibrator 1 > O.D. Kalibrator 2 > O.D. Kalibrator 3
O.D. Kalibrator 1 > O.D. Kalibrator 2 > O.D. Kalibrator 3

Die Charge wurde von der Qualitätskontrolle getestet und erfüllt alle Spezifikationen.
The lot has been tested by the quality control laboratory and meets the specifications.
## Anti-EBV-CA ELISA (IgG)
### Test instruction

<table>
<thead>
<tr>
<th>ORDER NO.</th>
<th>ANTIBODIES AGAINST</th>
<th>IG-CLASS</th>
<th>SUBSTRATE</th>
<th>FORMAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1 2791-9601 G</td>
<td>Epstein-Barr virus capsid antigen (EBV-CA)</td>
<td>IgG</td>
<td>Ag-coated microplate wells</td>
<td>96 x 01 (96)</td>
</tr>
</tbody>
</table>

**Principles of the test:** The ELISA test kit provides a semiquantitative or quantitative in vitro assay for human antibodies of the IgG class against EBV-CA in serum or plasma. The test kit contains microtiter strips each with 8 break-off reagent wells coated with EBV-CA. In the first reaction step, diluted patient samples are incubated in the wells. In the case of positive samples, specific IgG antibodies (also IgA and IgM) will bind to the antigens. To detect the bound antibodies, a second incubation is carried out using enzyme-labelled anti-human IgG (enzyme conjugate) catalysing a colour reaction.

### Contents of the test kit:

<table>
<thead>
<tr>
<th>Component</th>
<th>Colour</th>
<th>Format</th>
<th>Symbol</th>
</tr>
</thead>
</table>
| 1. Microplate wells  
coated with antigens: 12 microplate strips each containing 8 individual break-off wells in a frame, ready for use | —      | 12 x 8  | STRIPS |
| 2. Calibrator 1  
200 RU/ml (IgG, human), ready for use | dark red | 1 x 2.0 ml | CAL 1 |
| 3. Calibrator 2  
20 RU/ml (IgG, human), ready for use | red | 1 x 2.0 ml | CAL 2 |
| 4. Calibrator 3  
2 RU/ml (IgG, human), ready for use | light red | 1 x 2.0 ml | CAL 3 |
| 5. Positive control  
(IgG, human); ready for use | blue | 1 x 2.0 ml | POS CONTROL |
| 6. Negative control  
(IgG, human), ready for use | green | 1 x 2.0 ml | NEG CONTROL |
| 7. Enzyme conjugate  
peroxidase-labelled anti-human IgG (rabbit), ready for use | green | 1 x 12 ml | CONJUGATE |
| 8. Sample buffer  
ready for use | light blue | 1 x 100 ml | SAMPLE BUFFER |
| 9. Wash buffer  
10x concentrate | colourless | 1 x 100 ml | WASH BUFFER 10x |
| 10. Chromogen/substrate solution  
TMB/H₂O₂, ready for use | colourless | 1 x 12 ml | SUBSTRATE |
| 11. Stop solution  
0.5 M sulphuric acid, ready for use | colourless | 1 x 12 ml | STOP SOLUTION |
| 12. Test instruction | — | 1 booklet |
| 13. Quality control certificate | — | 1 protocol |

**Storage and stability:** The test kit has to be stored at a temperature between +2°C to +8°C. Do not freeze. Unopened, all test kit components are stable until the indicated expiry date.

**Waste disposal:** Patient samples, calibrators, controls and incubated microplate strips should be handled as infectious waste. All reagents must be disposed of in accordance with local disposal regulations.
Preparation and stability of the reagents

Note: All reagents must be brought to room temperature (+18°C to +25°C) approx. 30 minutes before use. After first use, the reagents are stable until the indicated expiry date if stored at +2°C to +8°C and protected from contamination, unless stated otherwise below.

- **Coated wells:** Ready for use. Tear open the resealable protective wrapping of the microplate at the recesses above the grip seam. Do not open until the microplate has reached room temperature to prevent the individual strips from moistening. Immediately replace the remaining wells of a partly used microplate in the protective wrapping and tightly seal with the integrated grip seam (Do not remove the desiccant bag). Once the protective wrapping has been opened for the first time, the wells coated with antigens can be stored in a dry place and at a temperature between +2°C and +8°C for 4 months.

- **Calibrators and controls:** Ready for use. The reagents must be mixed thoroughly before use.

- **Enzyme conjugate:** Ready for use. The enzyme conjugate must be mixed thoroughly before use.

- **Sample buffer:** Ready for use.

- **Wash buffer:** The wash buffer is a 10x concentrate. If crystallization occurs in the concentrated buffer, warm it to 37°C and mix well before diluting. The quantity required should be removed from the bottle using a clean pipette and diluted with deionized or distilled water (1 part reagent plus 9 parts distilled water).
  For example, for 1 microplate strip: 5 ml concentrate plus 45 ml water.
  The working strength wash buffer is stable for 4 weeks when stored at +2°C to +8°C and handled properly.

- **Chromogen/substrate solution:** Ready for use. Close the bottle immediately after use, as the contents are sensitive to light. The chromogen/substrate solution must be clear on use. Do not use the solution if it is blue coloured.

- **Stop solution:** Ready for use.

**Warning:** The control sera used have been tested negative for HBsAg, anti-HCV, anti-HIV-1 and anti-HIV-2 using enzyme immunoassays and indirect immunofluorescence methods. Nonetheless, all materials should be treated as being a potential infection hazard and should be handled with care. Some of the reagents contain the toxic agent sodium azide. Avoid skin contact.

Preparation and stability of the patient samples

**Sample material:** Human serum or EDTA, heparin or citrate plasma.

**Stability:** Patient samples to be investigated can generally be stored at +2°C to +8°C for up to 14 days. Diluted samples should be incubated within one working day.

**Sample dilution:** Patient samples are diluted 1:101 sample buffer. For example: dilute 10 µl serum in 1.0 ml sample buffer and mix well by vortexing (sample pipettes are not suitable for mixing).

**NOTE:** Calibrators and controls are prediluted and ready for use, do not dilute them.
Incubation

For **semiquantitative analysis** incubate calibrator 2 along with the positive and negative controls and patient samples. For **quantitative analysis** incubate calibrators 1, 2 and 3 along with the positive and negative controls and patient samples.

**(Partly) manual test performance**

**Sample incubation:**

(1°C)

Transfer 100 μl of the calibrators, positive and negative controls or diluted patient samples into the individual microplate wells according to the pipetting protocol. Incubate for **30 minutes** at room temperature (+18°C to +25°C).

**Washing:**

*Manual:* Empty the wells and subsequently wash 3 times using 300 μl of working strength wash buffer for each wash.

*Automatic:* Wash reagent wells 3 times with 450 μl working strength wash buffer (program setting: e.g. TECAN Columbus Washer "Overflow Modus").

Leave the wash buffer in each well for 30 to 60 seconds per washing cycle, then empty the wells. After washing (manual and automated tests), thoroughly dispose of all liquid from the microplate by tapping it on absorbent paper with the openings facing downwards to remove all residual wash buffer.

**Note:** Residual liquid (> 10 μl) remaining in the reagent wells after washing can interfere with the substrate and lead to false low extinction values. Insufficient washing (e.g., less than 3 wash cycles, too small wash buffer volumes, or too short reaction times) can lead to false high extinction values.

Free positions on the microplate strip should be filled with blank wells of the same plate format as that of the parameter to be investigated.

**Conjugate incubation:**

(2°C)

Pipette 100 μl of enzyme conjugate (peroxidase-labelled anti-human IgG) into each of the microplate wells. Incubate for **30 minutes** at room temperature (+18°C to 25°C).

**Washing:**

Empty the wells. Wash as described above.

**Substrate incubation:**

(3°C)

Pipette 100 μl of chromogen/substrate solution into each of the microplate wells. Incubate for **15 minutes** at room temperature (+18°C to 25°C) (protect from direct sunlight).

**Stopping the reaction:**

Pipette 100 μl of stop solution into each of the microplate wells in the same order and at the same speed as the chromogen/substrate solution was introduced.

**Measurement:**

**Photometric measurement** of the colour intensity should be made at a wavelength of 450 nm and a reference wavelength of between 620 nm and 650 nm within **30 minutes** of adding the stop solution. Prior to measuring, slightly shake the micro-plate to ensure a homogeneous distribution of the solution.

**Test performance using fully automated analysis devices**

Sample dilution and test performance are carried out fully automatically using the analysis device. The incubation conditions programmed in the respective software authorised by EUROIMMUN may deviate slightly from the specifications given in the ELISA test instruction. However, these conditions were validated in respect of the combination of the EUROIMMUN Analyzer I, Analyzer I-2P or the DSX from Dynex and this EUROIMMUN ELISA. Validation documents are available on inquiry.

Automated test performance using other fully automated, open system analysis devices is possible, however, the combination should be validated by the user.
The pipetting protocol for microtiter strips 1-4 is an example for the **semiquantitative analysis** of 24 patient sera (P 1 to P 24).

The pipetting protocol for microtiter strips 7-10 is an example for the **quantitative analysis** of 24 patient sera (P 1 to P 24).

The calibrators (C 1 to C 3), the positive (pos.) and negative (neg.) controls, and the patient samples have each been incubated in one well. The reliability of the ELISA test can be improved by duplicate determinations for each sample.

The reagent wells are break off format. Therefore, the number of tests performed can be matched to the number of samples, minimizing reagent wastage.

Both positive and negative controls serve as internal controls for the reliability of the test procedure. They should be assayed with each test run.

### Calculation of results

**Semiquantitative:** Results can be evaluated semiquantitatively by calculating a ratio of the extinction value of the control or patient sample over the extinction value of the calibrator 2. Calculate the ratio according to the following formula:

\[
\frac{\text{Extinction of the control or patient sample}}{\text{Extinction of calibrator 2}} = \text{Ratio}
\]

**EUROIMMUN** recommends interpreting results as follows:

- **Ratio < 0.8:** negative
- **Ratio ≥ 0.8 to < 1.1:** borderline
- **Ratio ≥ 1.1:** positive

In cases of borderline test results, an additional patient sample should be taken 7 days later and retested in parallel with the first patient sample. The results of both samples allow proper evaluation of titer changes.

**Quantitative:** The standard curve from which the concentration of antibodies in the patient samples can be taken is obtained by point-to-point plotting of the extinction values measured for the 3 calibration sera against the corresponding units (linear). Use “point-to-point” plotting for calculation of the standard curve by computer. The following plot is an example of a typical calibration curve. Please do not use this curve for the determination of antibody concentrations in patient samples.
If the extinction of a serum sample lies above the value of calibrator 1 (200 RU/ml). The result should be given as ">200 RU/ml". It is recommended that the sample be re-tested at a dilution of 1:400. The result in RU/ml read from the calibration curve for this sample must then be multiplied by a factor of 4.

The upper limit of the normal range of non-infected persons (cut-off value) recommended by EUROIMMUN is 20 relative units (RU)/ml. EUROIMMUN recommends interpreting results as follows:

- <16 RU/ml: negative
- ≥16 to <22 RU/ml: borderline
- ≥22 RU/ml: positive

For duplicate determinations the mean of the two values should be taken. If the two values deviate substantially from one another the sample should be retested.

For diagnosis, the clinical symptoms of the patient should always be taken into account along with the serological results.

**Test characteristics**

**Calibration**: As no international reference serum exists for antibodies against EBV-CA, the calibration is performed in relative units (RU).

For every group of tests performed, the extinction values of the calibrators and the relative units and/or ratios determined for the positive and negative controls must lie within the limits stated for the relevant test kit lot. A quality control certificate containing these reference values is included. If the values specified for the controls are not achieved, the test results may be inaccurate and the test should be repeated.

The activity of the enzyme used is temperature-dependent and the extinction values may vary if a thermostat is not used. The higher the room temperature during substrate incubation, the greater will be the extinction values. Corresponding variations apply also to the incubation times. However, the calibration sera are subject to the same influences, with the result that such variations will be largely compensated in the calculation of the result.

**Antigen**: The microplate wells were coated with the purified Epstein-Barr virus capsid antigens. The antigen source is provided by inactivated cell lysates of human B cells infected with the "P3HR1" strain of Epstein-Barr viruses.
Linearity: The linearity of the Anti-EBV-CA ELISA (IgG) was determined by assaying 4 serial dilutions of different patient samples. The coefficient of determination R² for all sera was > 0.95. The Anti-EBV-CA ELISA (IgG) is linear at least in the tested concentration range (4 RU/ml to 141 RU/ml).

Detection limit: The lower detection limit is defined as the mean value of an analyte-free sample plus three times the standard deviation and is the smallest detectable antibody titer. The detection limit of the Anti-EBV-CA ELISA (IgG) is 0.9 RU/ml.

Cross reactivity: The quality of the antigen and the source of antigen (P3HR1-Cells EBV infected) used ensures a high specificity of the ELISA. No cross reactivities with Herpes viruses were determined. The test is anti-EBV specific.

<table>
<thead>
<tr>
<th>Antibodies against</th>
<th>n</th>
<th>Anti-EBV-CA ELISA (IgG)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus</td>
<td>10</td>
<td>0%</td>
</tr>
<tr>
<td>Chlamydia pneumoniae</td>
<td>5</td>
<td>0%</td>
</tr>
<tr>
<td>CMV</td>
<td>3</td>
<td>0%</td>
</tr>
<tr>
<td>Influenza virus A</td>
<td>4</td>
<td>0%</td>
</tr>
<tr>
<td>Influenza virus B</td>
<td>9</td>
<td>0%</td>
</tr>
<tr>
<td>Measles virus</td>
<td>9</td>
<td>0%</td>
</tr>
<tr>
<td>Mumps virus</td>
<td>9</td>
<td>0%</td>
</tr>
<tr>
<td>Mycoplasma pneumoniae</td>
<td>3</td>
<td>0%</td>
</tr>
<tr>
<td>Parainfluenza virus Pool</td>
<td>10</td>
<td>0%</td>
</tr>
<tr>
<td>RSV</td>
<td>8</td>
<td>0%</td>
</tr>
<tr>
<td>Rubella virus</td>
<td>10</td>
<td>0%</td>
</tr>
<tr>
<td>VZV</td>
<td>5</td>
<td>0%</td>
</tr>
</tbody>
</table>

Interference: Haemolytic, lipaemic and icteric samples showed no influence at the result up to a concentration of 10 mg/ml for hemoglobin, 20 mg/ml for triglycerides and 0.4 mg/ml for bilirubin in this ELISA.

Reproducibility: The reproducibility of the test was investigated by determining the intra- and inter-assay coefficients of variation using 3 sera. The intra-assay CVs are based on 20 determinations and the inter-assay CVs on 4 determinations performed in 6 different test runs.

<table>
<thead>
<tr>
<th>intra-assay variation, n = 20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>inter-assay variation, n = 4 x 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
</tbody>
</table>

Specificity and sensitivity: 111 clinically and serologically precharacterized sera (Interlaboratory test samples of INSTAND, Germany / Labquality, Finland) were examined with this EUROIMMUN ELISA. The test showed a specificity and a sensitivity of 100% each.

<table>
<thead>
<tr>
<th>n = 111</th>
</tr>
</thead>
<tbody>
<tr>
<td>INSTAND / Labquality</td>
</tr>
<tr>
<td>positive</td>
</tr>
<tr>
<td>EUROIMMUN Anti-EBV-CA-ELISA (IgG)</td>
</tr>
<tr>
<td>positive</td>
</tr>
<tr>
<td>negative</td>
</tr>
</tbody>
</table>

Reference range: The levels of the anti-EBV-CA antibodies (IgG) were analyzed with this EUROIMMUN ELISA in a collective of 500 healthy blood donors. With a cut-off of 20 RU/ml, 93.4% of the blood donors were anti-EBV-CA positive (IgG) which reflects the known percentage of infections in adults.
Clinical significance

Epstein-Barr virus (EBV) and herpes simples virus types 1 and 2 belong to the most ubiquitous human herpes viruses in adults [1]. Epstein-Barr virus is the causative agent of infectious mononucleosis (glandular fever), a febrile disease usually accompanied by pharyngitis and lymphadenopathy, frequently by hepatosplenomegaly and more rarely by rash, exantheme. EBV infections are also found in connection with the pathogenesis of Burkitt's lymphoma, nasopharyngeal carcinoma, and, as current research results show, multiple sclerosis [1, 2, 3, 4, 5, 6, 7, 8]. Infectious mononucleosis must be differentiated from cytomegalic inclusion body disease and toxoplasmosis and, in the case of atypical progress, also from HIV or other infections [1, 9, 10].

In pregnancy Epstein-Barr virus can cause infection of the placenta with damage to the foetal heart, eye and liver [3, 11]. In children, accompanying infections of the kidney have been observed with symptoms from microscopic haematuria to acute kidney failure [1, 3, 12].

The immune response to an EBV infection is characterised by formation of antibodies against the EBV capsid antigen (EBV-CA), against EBV nuclear antigens (EBNA-1 to EBNA-6) and against EBV early antigens (EBV-EA) [1, 2, 3, 4, 5, 6, 7, 8, 13, 14, 15, 16, 17, 18, 19].

In 90% of cases an acute EBV infection can be characterised serologically by the detection of EBV-CA IgM and an increase in titre of EBV-CA IgG using ELISA [13, 14, 15, 16].

A titer increase of at least two-fold for anti-EBV-CA IgG with simultaneous lack of antibodies against EBNA-1 demonstrates the early phase of an acute EBV infection [16]. Of importance in this respect is that in an EBV infection EBNA-1 to EBNA-6 are synthesised earlier than the other EBV antigens (EBV-CA and EBV-EA), but they are only presented to the immune system after the destruction of B cells, so that, timewise, antibodies against EBV-CA and EBV-EA are detectable before antibodies against EBNA [6, 19, 20].

An IgM immune response to EBV-CA with a titer increase of EBV-CA IgG antibodies is considered a reliable indicator of the presence of an acute EBV infection [13, 15, 17]. An early EBV infection can be confirmed using EBV-CA IgG avidity determination [13].

IgA antibodies against early EBV proteins are detectable in primary infections, and rarely in reactivations [6]. IgG antibodies against early EBV proteins occur in 70% to 80% of patients with infectious mononucleosis, although only temporarily during the acute phase.

High antibody titers of class IgA against EBV-CA, and also class IgG against EBV-EA can be evaluated as an indication of Burkitt's lymphoma or nasopharyngeal carcinoma, which extends the diagnostic potential of this test [1, 3, 4, 5, 6, 7, 8, 18].

In considering the significance of the detection of antibodies against EBV-EA, it should be taken into account that these can, but don't have to, occur in acute infections and during an inapparent disease course. Differentiation between a primary EBV infection and the more rare reactivation is hardly possible serologically [13, 15, 19].

With the ELISA test for the quantitative in vitro determination of human IgG antibodies against EBV-CA in cerebrospinal fluid (CSF) the CSF/serum quotient of the agent-specific antibodies CSQ$_{\text{spec.}}$ (IgG) can be measured. The quotient can indicate EBV antibody production in the central nervous system and thus enable diagnosis of a cerebral EBV infection [21].


