بسم الله الرحمن الرحيم

Sudan University of Science and Technology

Collage of Graduate studies

Sero-detection of Epstein Barr Virus IgGAntibodies amongLeukemic Patients in Khartoum State

الكشف المصلي للاجسام المضادة لفيروس ابشتاين بار لدى مرضى سرطان الدم بولاية الخرطوم

A dissertation submitted in Partial fulfillment for the requirement of M.Sc degree in Medical Laboratory Sciences (Microbiology)

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بسم الله الرحمن الرحيم

الآية

قال الله تعالي :

وَلَوْلاَ فَضْلُ اللَّهِ عَلَيْكَ وَرَحْمَتُهُ لَهَمَّت طَّائِفَةٌ مِّنْهُمْ أَن يُضِلُّوكَ وَمَا يُضِلُّونَ إِلاَّ أَنفُسَهُمْ وَمَا يَضُرُّونَكَ مِن شَيْءٍ وَأَنزَلَ اللَّهُ عَلَيْكَ الْكِتَابَ وَالْحِكْمَةَ وَعَلَّمَكَ مَا لَمْ تَكُنْ تَعْلَمُ وَكَانَ فَضْلُ اللَّهِ عَلَيْكَ عَظِيمًا)

سورة النساء الآيه (113)

صدق الله العظيم

Dedication

I dedicate this work to my parents for their kind auspice and to my all friends who offer me great help to accomplish this work.

Acknowledgement

First of all a great thanksto ALMIGHTY ALLAH for helping me to finish this study.

Of course a great appreciation to Prof. YousifFadllafor his invaluable advice and guidance.

I owe deep gratitude to several persons for their substantial help namely the staff ofRadiation Center (RICK),I also owe a great debt to the colleagues in the NationalHealth Public Laboratory ,Department of clinical Isotope

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Abstract

The main aim of this study was to determine the sero prevalence of Epstein-Barr virus (EBV) IgG antibody using enzyme linked immunosorbent assay (ELISA) among leukemic patient attending Radiation Isotop-center-Khartoum (RICK)from April to June 2015

This study had also examined age, gender and type of leukemia antibodies as risk factors.

A total of 80 subjectsof leukemicpatients were examined.

The results revealed that the prevalence of Epstein-Barr virus (EBV)IgG among total leukemic patients was 60(75%)

The results showedthat the prevalence of Epstein-Barr virus infectionwas21 (23%) in patient withacute lymphoid leukemia(ALL), 13 (14%)inchronic Leukemia(CLL), 8(8.9%) acute myeloid leukemia (AML) and 18(20%. chronic myeloid leukemia (CML) .According to gender it was 34(42.5%) in males,in females 26(32.5%).

Also the results showed high occurance of EBV IgG antibodies in age groupsbetween (1-20)years which represent21(26.3%), followed by the age groups between(41-60) years which represent 19(23.8%) and then age groups between (21-40)years represent 16 (20.0%),and less occurance in age groups between (61-80)which resemble 4(4%).

From this Study concluded that there is significant correlation between leukemia and Epstein-Barr virus .The study showed that EBV was one of the causes of leukemia and recommended further studies to enhance these results.

المستخلص

الهدف الرئيسي من هذه الدراسة هو تحديد مدي انتشار الاجسام المضاده من النوع IgGلفيروس ابشتاين بارباستخدام مقايسة الامتصاص المناعي المرتبط بالانزيم (اليزا (ELISA) بين مرضى اللوكيميا(سرطان الدم)بالمركز القومي للعلاج بالاشعاع والطب النووي Radiation Isotop-center-Khartoumبولاية الخرطوم في الفتره مابين ابريل الى يونيو من العام2015وقد شملت هذه الدراسه 80 شخصا من مرضى سرطان الدم.وقد بحثت هذه الدراسة أيضا العوامل التي تزيد من معدل الاصابه بفيروس ابشتاين بار مثل العمر،النوع،الجنس ونوع من سرطانالدم على الأجسام المضادة.

وكشفت النتائج أن معدل انتشار الاجسام المضاده لفيروس ابشتاين بار بين مجموع العينات كانت (75.0%)60عند مرضي الليوكيميا(سرطان الدم) .

وأظهرت النتائج زياده في معدل انتشار فيروس ابشتاين بار الأجسام المضادة من النوع IgGلسرطان الدم اللمفاوي الحاد.وكانت النتائج المتحصل عليها في سرطان الدم اللمفاوي الحاد 21 (23٪)وكانت في سرطان الدم الليمفاوي المزمن13 (14٪)، سرطان الدم النخاعي الحاد 8 (8.9٪)وسرطان الدم النخاعي المزمن 18 (20٪).

كما أظهرت النتائج بالنسبه للنوع،عندالزكور(42.5%) 34،وعندالإناث (32.5%)26كما اظهرت النتائج ايضا زياده في معدل انتشار الاجسام المضاده لفيروس ابشتاين في الفئه العمريه مابين (21-1) سنه حيث تمثل (26.3%) 21 تليها الفئه العمريه مابين(60-41) تمثل نسبة (23.8%)19 ومن ثم الفئه العمريه من(40-21) تمثل نسبة (20.0%)16s بينما مثلت اقل نسبه في الفئه العمريه مابين(80-61)حيث مثلت 4(4%) .

وخلصت هذه الدراسه الي العثور على وجود علاقة ذات دلالة إحصائية بين سرطان الدم وفيروس ابشتاين بار. كما أظهرت الدراسه أن فيروس ابشتاين هو احد أسباب سرطان الدم، وأوصتباجراء مزيد من الدراسات لتعزيز هذه النتائج .

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Abbreviations

List of abbreviations

Abbreviations	Complete word
AIDS	Acquired immunodeficiency Syndrome
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
CLL	Chronic lymphocytic leukemia
CML	Chronic myeloid leukemia
DNA	Deoxyribo nucleic acid
EA	Early antigen
EBNA	Epstein –Barr Nuclear antigen
EBV	Epstein –Barr Virus
ELISA	Enzyme- linked immunosorbent assay
HH-7	Human Herpes-7
HH-8	Human Herpes-8
HH-4	Human herpes virus-4
HIV	Human Immuno deficiency Virus
HL	Hodgkin lymphoma
IF	Immunoflourescent
IgA	Immunoglobuin A
IgG	Immunoglobuin G
IL-10	Interleukin-10
LCL'S	Lymphoblastoid cell lines
LMP	Latent membrane protein
NK	Natural Killer Cell

NPC	Nasopharyngeal carcinoma
ORF	open reading frame
PCR	Polymerse chain reaction
PEL	Primary effusion lymphoma
PTLD	Posttransplantlymphoproliferative disorder
TH-1	T helper-1
VCA	Viral capsid antigen
XLP	X-linkedlymphoproliferative syndrome

CHAPTER ONE

CHAPTER ONE

1. Introduction

1.1Introduction

Epstein-Barr virus(EBV) is a member of the herpesvirus family. As with other herpes viruses, EBV is an enveloped virus that contain DNA core surrounded by an icosahedral nucleocapsid and tegument .Family members include herpes simplex I and II and varicella zoster virus (alpha-herpesvirus subfamily),cytomegalovirus and human herpes virus(HHV-6) and(HHV-7) (beta-herpesvirus subfamily) and (HHV-8) and EBV(gamma-herpesvirus subfamily).(Young and Rickinson .,2004).

Human tumors have been attributed to both HH-8(Kaposi sarcoma),primary effusion lymphoma(PEL) and EBV(Burkitts lymphoma,nasophrangeal carcinoma ,and Hodgkin's lymphoma)and non-Hodgkin's lymphoma). (Chen *et al*., 2007)

EBV has a world-wid distribution being able to establish a long life infection is more than 90% of individuals. Primary infection is usually asymptomaticand only when it delays until adulthooda being lymphoproliferative disease known as infectious mononucleosis(IM),may occur. The main site of EBV persistence in vivo is represented by latently infected B-cell showing feature of resting memory B lymphocyte in vitro.(Miyashita*etal*.,1997)

The presence of EBV genomes and constant expression of viral protein, strongly support the involvement of EBV in the pathogensis of awide spectrum of human malignancies. These include lymphomas of B,T and NK cell origin such as the immoblastic lymphoma of immuno suppressed,endemic Burrkitt's lymphoma(BL),Hodgkin'slymphoma(HL).But also carcinoma of thenasopharynx and leiomyosacoma arising in organ transplant patient

and HIV infected individuals.(Chen *et al.,* 2007).This work was carried out to find on association between EBV infection and leukemia.

2.1 Rationale

Epstein-Barr virus ,awidely distributed herpes virus that associated with several malignant diseases.The major target cell for EBV is the B-lymphocytebut can also infect other cell lymphoproliferative disorders that have been reported to be EBV associated include a subset of peripheral T-cell lymphomas, angioimmunoblastic T-cell lymphoma (AILT), extranodal nasal type NK/T-cell lymphoma,enteropathy-type T-cell lymphoma, γδ T-cell lymphomas (hepatosplenic and nonhepatosplenic), T-celllymphoproliferative disorders after chronic EBV infection, EBV-associated cutaneousT-cell lymphoproliferative disorders and aggressive NK-cell leukemia/lymphoma (Rezk and Weiss., 2007)Epstein Barr Virus associated with acute Lymphocytic leukemia in childhood(Sehgal*etal*.,2010).

EBV also associated with well defined group of lymphoid and epithelialtumar in which thevirus directly drives transformation of infected cells.Recent evidence however indicates that this virus may infect a sub population oftumar cells in patient with chronic lymphocytic leukemia (CLL).

EBV is one of the serious virus in sudanamong adults and child,among childhood observed with high incidence Of EBV with acute leukemia.(Hussein *et al.,*2011). According to (Elwad*et al.,*2014) there is on evidence of the involvement of EBV in patients with leukemia.

1.3Objectives

1.3.1General objective

To investigate the seroprevalence ofEpstein Barr Virus antibodies among leukemic patients areattendingRadiationIsoptop Center-Khatoumstate(RICK).

1.3.2Specific objective

1-Todetect the specific Epstein Barr Virus IgG(VCA)in serum obtained from leukemic patient s using enzymelinkedimmunosorbentassay(ELISA) .

2-To find out the major risk factor associated with EBV infection among leukemias.

3-To compare findingbetweenLymphobastic leukemia (Acute lymphoblastic leukemia (ALL),Chronic lymphoblastic leukemia(CLL))and myeloid leukemia(Acute myeloid leukemia (AML), Chronic myeloid leukemia (CML)).

CHAPTER TWO

CHAPTER TWO

2.Literature review

2.1Historical background

EBV was discovered as a result of pioneering work in the 1950 ,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 (known as Burkitt's lymphoma) appeared to be influenced by climatic factors notably temperature and elevation.

Epstein and Yvonne Barr were the first identify EBV in tumor tissue associated with Burkitt'sLymphoma. The researchers used electron microscopy to determine that these viral particles were very similar in structure to Herpes simplexvirions (Epstein *et al*.,1964).

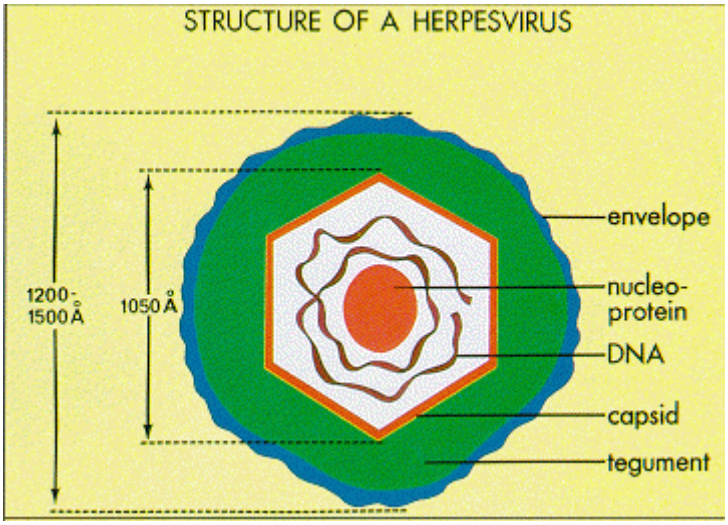
2.2Virusclassification and Genome

The Epstein-Barr virus is a Baltimore Class I virus of the familyHerpesviridae. It is a gammaretrovirus also designated as Human Herpes Virus 4 (HHV-4). The genome of EBV is composed of double-stranded DNA and is 172,282 base-pairs long(Baer *etal*.,1984) . This relatively long genome is characteristic of Herpes viruses; Herpes simplex has a 152-kilobase genome(Mahiet*et al*.,2012). The open reading frames (ORFs) or EBV are generally broken up into separate lytic and latent sections . While most of the viral genes encode proteins, some of the latent genes remain untranslated (EBERs: ENV encoded RNAs) and several micro-RNAs are coded for(Mahiet*etal*., 2012). The relativity large dsDNA genome of the Epstein-Barr virus exhibits separation of ORF sections coding for genes associated with its two different

replicativestrategies.During latent infection, the EBV genome exists in a circularized form localized in the host cell nucleus(Young and Rickinson.,2004)The reading frames for the LMP and EBNA proteins are clustered separately

2.3Epstein-Barr virus-Virion structure

EBV-observed that this lymphoma-associated virus was about20% smaller than typical Herpes simplex virion(Epstein *et al*.,1964) .Similar to other Herpesvirus,the inner most part of the EBV virion consist of DNA wrapped around a centeral a nucleocapsid,alyer of protein tegument,and finallyand outer envelope with spike glycoprotein(LiebowitzandKieff.,1993)See (figureA) Similar to many other viruses,many of these glycoprotein are vital for host cell entry mechanism. Infected host cells release EBV virionexclusively during the lytic cycle.



Figure(A). A general diagram of a herpes virusvirion. Note that the dsDNA genome is wrapped around a central nucleo-protein. Spike.glycoprotein on the surface play a role in host cell entry (Young and Rickinson .,2004)

2.4Strains of Epstein-Barr virus There are two strains of EBV,EBV -1and EBV-2,which differ in the regions that code for the EBNAs and EBERs.EBV-2 is less effective at causing B-cell growth and proliferation than EBV-1

This is mainly a result of differences in the coding regionof thegene for EBNA2 (Baumforth et *al*.,1999l).

2.5 Transmission of infection

EBV transmission take place through oropharyngeal secretion .In adolescent and adult cases of infectious mononucleosis intimate kissing has been the main route of transmission whereas saliva.(Sawyer *et al*.,1971). EBV infection frequently takes place among smaller children of low socio economic status,in nurseries and when sharing a room (Crawford *et al*., 2002)

EBV has been detected in cervical secretions in between 8 and 28% of teenage girls and adult women,and in semen samples in men (Enbom*et al*.,2001),but evidence on whether EBV is transmitted through genital contact is limited.

EBV may be spread through blood transfusion an as a result of organ transplantation(Scheenstra*et al*.,2004).

Transmission is of particular concern in association with organ transplantation where primary EBV infection is amajor risk factor for post-transplant lymphoproliferativedisease(PTLD) (Scheenstra*et al.,* 2004).

2.6Epidemiology of Epstein-Barr virus

Endemic Burkitt's lymphoma occurs frequently in young children in the equatorial regions of Africa and Papua New Guinea and has an incidence of 50-100 cases per 1,000,000 individuals (Kutok and Wang.,2006). In contrast, EBV-associated sporadic lymphoma occurs in children and young adults and has no specific geographic distribution, with an incidence of 2-3 cases per 1,000,000 individual. It accounts for 40 and 50% of childhood non-Hodgkin's lymphomas (NHLs) and 1-2% of adult lymphomas in Western Europe and the United States(Yusteinand Dang*etal*., 2007). Nasopharyngeal carcinoma (NPC) is most common in southern China, and accounts for approximately 20% of all adult cancers (Shah and Young*etal.,*2009). It is extremely rare in Europe and North America, with an incidence rate is <1 per 100,000 population.

2.7 life cycle

Knowledge of the EBV life cycle is important to better understand clinical symptoms andEBV diagnostics. The 186-kb double-stranded DNA EBV genome codes for a number of structural and nonstructural genes. The port of entry for EBV is also the port of exit, i.e; the oropharynx. After entry, EBV replicates in epithelial cells and B- cells in the oropharynx and spreads through the body via infected B cells, while latent genes that either drive B cells to EBV lytic cycle entry or acquire the status of latency are differentially expressed (Borza and Fletcher., 2002) . Like other herpesviruses,EBV follows a The latent infection is established by self-replicating extrachromosomal nucleic acid, the episomes (Crawford *eta.,*2002) . The virus is intermittently shed from saliva; thus, the main route of transmission is directly from person to person; however, transmission via blood products, transplantation, and sexual transmission were shown Interestingly, found that healthy

individuals may be infected with multiple different EBV genotypes, in which the sequences of the open reading frames encoding EBV nuclear antigens (EBNAs) differ among the different genotypes (Crawford *et al.,*2002) Taken together, the prevention of virus spread seems impossible.EBV transforms B cells in vivo (in the body) and in vitro (in cell culture), thus immortalizing B cells.

2.8 EpsteinBarr Virus Lytic Cycle

While the EBV lytic life cycle is more rarely observed than it is the only way that the virus may make virions and be transferred horizontally between hosts (or cells) (LiebowitzandKieff.,1993) Immunosuppressive diseases like AIDS typically show increased free virion levels in the blood, a marker of increased lytic activity. While virionsare often found in the saliva of infected hosts, little to no lytic-infected cells are typically detected in the body(Swamminathanand Kenny.,2009). Human cytotoxic T-cells are particularly adept at recognizing and destroying lyticallyinfected cells expressing certain early stage lytic genes(Steven*etal*.,1997). Hence, lytic activity appears to drive EBV spread in human populations. However, the latent replicative cycle is favored under normal conditions within a host, possibly as a means to evade host immune responses. Alpha and Beta herpesviruses have elaborate mechanisms for lytic gene concealment from the host immune system while EBV has few mechanisms to prevent immune mediated destruction of lytic-infected cells For example, Herpes simplex may be capable of inhibition of host.MajorHistocombatibility Complex, which reduces B-cell antigen presentation and recognition by cytotoxic T-cells(Steven*etal*.,1997). In contrast to other herpesviruses, gammaherpesvirusessuch as EBV have a different mechanism for avoidance of host immune response. EBV primarily relies on a latent replication cycle in which its genome proliferates via clonal replication

within dividing host B-cells .Hence, unlike other herpesviruses that rely on lytic replication for spread within a avoidance traits. Host, EBV relies more on its latent mechanism. Virion production by EBV takes on a specific "niche role" vital for the initial infection of a new human host. Because latent mechanisms are responsible for sustained infections, EBV is not under significant selective pressure to "develop" elaborate lytic immune. The lytic cycle begins with mature EBV virions reaching target host cells, such as B-cells. Contact between the virion and B-cell is initiated by the binding glycoprotein 350 to B-cell of EBV CD-21(Nemerow*et al.*,1989) For the most part, EBVinfection is specific to cells expressing membranous CD-21 (namely B-cells and some epithelia), though some CD-21 independent attachment mechanisms are possible albeit with low efficacy (LiebowitzandKieff.,1993)EBV 350 is an example of a lytically expressed gene that is specifically targeted by the human immune system This gp350 binding is complemented by the binding of EBV gp42 to B-cell MHC-II(Borza and Fletcher.,2002).In order for the viral envelope and B-cell membrane to fuse, the EBV virion must have functional gH, gL, and gp42 to B-Cell MHCII-I

Once the gH/gL/gp42 complex penetrates the B-cell membrane, the viral capsid is dissolved, allowing the viral dsDNA genome to enter the cytoplasm. Interesting, viral, viral dsDNA generally localizes to the nucleus forreplication*(*Daikoku*et al*., 2005*)*. Viral dsDNA is replicated using host machinery, which stimulates the production of viral structural proteins. Viral particles are then assembled in the nucleus (Swaminathanand Kenny .,2009). After full particles are assembled, they bud out of the nuclear membrane, then the cell membrane. When exiting the host cell, these virions may acquire their primary envelope from the nuclear membrane, and the outer envelope from the cell membrane (Gong

and Kieff.,1990).Unlike many other herpesviruses, this exit mechanism does not initiate mandatory cell death. In the lytic life cycle, viral ds DNA generally localizes to the nucleus for replication (Daikoku *et al*.,2005). Viral dsDNAisreplicated using host machinery, which stimulates the production of viral structural proteins. Viral particles are then assembled in the nucleus(Henson *etal*.,2009). After full particles are assembled, they bud out of the nuclear membrane, then the cell membrane.

2.9Latent life cycle

Thelatent life cycle makes a moredirectcontribution to lymphoproliferative begins at either the Wp or Cp promoter differential splicing disease. In most cases, once EBV virions achieve primary infection of B-lymphocytes, the virus primarily replicates by a latent mechanism (Jochum*etal.,*2012). This results in the transformation of B-cells into ever-proliferating lymphoblastoid cell lines (LCL's) (Young and Rickinson.,2004). The latent phase in the EBV viral life cycle is defined by:((1)No production of virions, and (2) the production of a select few viral proteins and transcripts (Cesarman.,2011)). Many of these select latent viral proteins modulate the character of host B-cells and contribute to lymphoproliferation(Cesarman.,2011) .In contrast to the lytic cycle where the viral genome is brought to the nucleus and copied, under the lytic cycle infected B-cells contain several copies of extrachromosomal EBV episomes (Young and Rickinson.,2004)Hence, as the B-cells proliferate, so does thevirusLatency can be divided into 3 distinct stages defined by which latent genes are being expressed In latency I, only EBNA1 is expressed(Cesarman.,2011), while in latency II EBNA1 is expressed along with intermediate levels of other EBNAs and LMP proteins (Youngand Rickinson.,2004). Latency II can be subdivided into

IIa, where EBNA2, but not LMP1 is expressed, and IIb, where the expression of these two proteins is reversed(Cesarman., 2011) .Latency III is characterized by unregulated expression of the complete latent viral proteome which includes EBNAs 1-6, as well as 3 LMP proteins (1,2A, and 2B(Cesarman., 2011) . B-cells must have latency III expression profiles for successful generation of LCLs in vitro (Klein., 2007). In addition to these viral proteins, several non-coding RNAs (EBERs) and micro-RNAs are also produced during all three stages of latency (Fok*et al*.,2006)

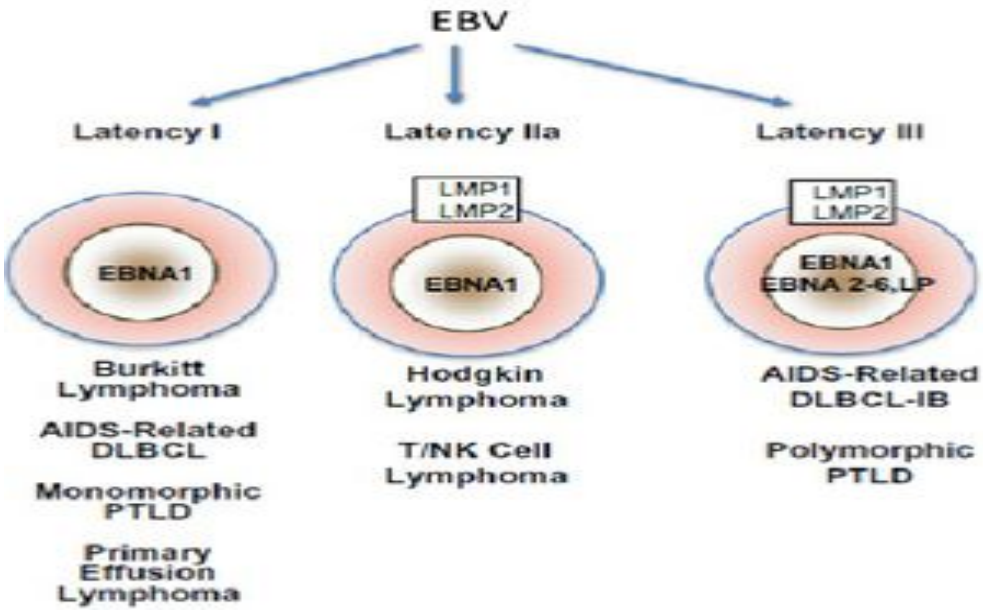


Figure B. The spectrum of viral latent gene expression in EBV infected B-cells. Below the schematic for each latency pattern are the lymphoproliferative disorders associated with each type of expression. Source:(Cesarman*etal*.,2011)

2.10 Pathogenesis of EpsteinBarr virus

After infecting the oropharynx tissue such as the salivary gland,EBV then infects and transforms B-cells s into B -cells withunilimited growth potential. Cytotoxic T-cells and Natural Killer cells are then activated in

defense against EBV and regulate the elimination of infected B-cell. In extreme case when long lasting infection results,agammaglobulinemia, the lack of gamma globulin occur due to T cells severely suppressing the B cells. in such cases chronic EBV syndrome observed, and symptoms from EBV last for up to six months due to the severity of B-cell suppression .At this time other infection can occurring infections are treated and eventually the patient recovers.If the T-cell response is not strong lymphocytes occurs resulting in alymphoma.Normally an EBV infection can occur,and patient observes the usual symptoms such as fever and swollen lymphatic tissue that result from the immune system fitting the proliferation of infected B-cell until full recovery is accomplished (O.R.R.,1981).

2.11 Clinical significance

Most primary infections in children are asymptomaticIn adolescents and young adults, the classic syndrome associated with primary infection in infectious mononucleosis(50% of infection).EBV also is associated with several types of cancer.(Geo *etal.,* 2013).

2.11.1 Infectious mononucleosis

After an incubation period of 30-50 days, symptoms of head-ache,fever , malaise, fatigue and sore throat occur. Enlaged lymph node and spleen are characteristic. Some patients develop signs of hepatitis.

The typical illness is self-limited and lasts for 2-4 weeks. During the disease,there is an increase in the number of circulating white blood cells, with predominanceof lymphocytes.Many of these are large,atypical T lymphocytes.Low-grade fever and malaise may persist for weeks to months after acute illness. Complications are rare in normal hosts.(Geo *et al*., 2013).

2.11.2EpsteinBarr virus and malignant disease

EBV associated with several type cancersuch as nasopharyngeal carcinoma,Hodgkin lymphomas ,and Gastric carcinoma(Geo*et al*.,2013)

2.11.2.1Burkitt's Lymphoma

Burkitt's lymphoma is a highly proliferative B-cell tumor that includes three variants: endemic (affecting children in equatorial Africa and New Guinea), sporadic (affecting children and young adults throughout the world) and immunodeficiency related (primarily in association with HIV infection). EBV has been detected in virtually all cases of the endemic variant, 15%–20% of cases of the sporadic variant, and 30%–40% of cases of the immunodeficiency-related variant (Young and Rickinson.,2004)

2.11.2EpsteinBarr Virus Associated Lymphomas inimmunocomporomised individuals

Thereexist several distinctclasses ofEB-associatedlymphoproliferative disorders in immune compromised individuals. First, there is a disorder resulting from an inherited immunodeficiency known as X-linked lymphoproliferative disorder. Second, there are lymphomas associated with immunosuppressive drugs given to transplant recipients. Finally, there are AIDS-relatedlymphoproliferative disorders. The most common gene-expression pattern in these disorders is latency III.

2.11.2.3Nasopharyngeal Carcinoma(NPC)

A cancer of epithelial cells and common in males of Chinese origin.EBV DNA is regularly found in nasopharyngeal carcinoma cells, and patient

have high levels of antibody to EBV. EBNA1 and LMP1 are expressed. Genetic and environmental factors are believed to be important in the development of nasopharyngeal carcinoma(Geo *etal*.,2013)

2.11.3 X-linked lymphoproliferative syndrome

X-linked lymphoproliferative syndrome (XLP) or Duncan's disease is a rare, primary immunodeficiency that was first described as a familial disorder affecting males with a rapidly fatal course in response to EBV infection (Purtilo*et al*., 1975). XLP is characterized by three major phenotypes (fulminant infectious mononucleosis, B-cell lymphoma and dysgammaglobulinemia

2.11.4 Post-transplant lymphoproliferative disease

"Post-transplantlymphoproliferative disorder" (PTLD) in reality defines a spectrum of lymphoid hyperproliferative states that may be observed in solid organ and bone marrow transplant recipients (Loren *et al* .,2003). It has been suggested that EBV-associated PTLD should include two of the following three features: disruption of underlying architecture by, presence of monoclonal or polyclonal cell populations, and evidence (typically by in situ hybridization for virus-encoded RNA) of EBV in many of the cells (Paya*et al.,*1999)

2.12Immune system react with EpsteinBarr virus

Cytotoxic CD8 T-Cells specific for a certain EBV antigen find B-Cells bearing their antigen and kill them; NK Cells can also kill EBV infected B-Cells. During the latent stage, CD8 T-Cells for latent proteins regulate the amount of latent protein expressing EBV infected B-Cells also. (Steven *et al* .,1997).

Constant exposure to IL-10, as seen in patients with chronic EBVinfections, results in anti-IL-10 antibodies being made. These antibodies are hypothesized to be produced by CD5 B-Cells which are known to be the source of autoantibodies such as ones against IL-10, and CD5 B-Cells have been shown to increase by three fold during an EBV infection. Thus the immune system fights back with anti-IL-10 antibodies to unsuppress macrophage activation. (Tanner*et al*.,1997) .

Infected B-Cells can be caused to undergo CD95-CD95L-mediated apoptosis (Fas/FasL apoptosis) by CD4 T-Cells. Some TH1 CD4 T-Cells bear CD95L and can bind to EBV infected B-Cells bearing the CD95 receptor ultimately resulting in apoptosis. This normal mechanism of regulation of B-Cell growth is observed during EBV infection (Wilson *et al*., 1998).

2.13 Serologic Response of patient with Epstein –Barr virus-associated disease

Immunoglobulin M anti bodies to EBV viral capsid antigen (anti-VCA) evolve quickly with infection, persist for weeks to month and do not re appear .Thus their detection is presumptive evidence of recent primary infection.Analysis of paired acute and convalescent sera show arise a subsequent fall ,and along life persistence of IgG anti VCA.

IgA antiVCA is not useful except in the diagnosis and management of nasopharyngeal carcinoma.

Antibodies to viral early (pre-DNA synthesis)antigens (anti –EA)of the diffuse or restricted types develop in most primary infections.Its no diagnostic significant.(Horwitz*et al*.,1985).

Antibodies to EBV nuclear antigen (anti-EBNA) are detected in traditional assays relativelylate afterthe onset of symptoms in infectious mononucleosis so that their absence in apreviously well person who develops acute illness suggest an ongoing EBV infection.

2.14 laboratory Diagnosis

Symptoms of an EBV infection resulting in mononucleosis can mislead diagnosis. So may resemble other infections such as sore throat strep infection.So the diagnosis includes:

-Hetrophil antibody test or(Paul-Bunnell test).

Used to detect EBV infection. heterophil antibodies have ability to clot sheep or horse red blood cells,a small amount of the patient's blood is added to some blood to see clotting occurs,but this method misleading for younger children so do not produce enough heterophil antibody .Also other infections can produce heterophil antibodies as well as mislead diagnosis.(O.R.R .,1981).

-EBV-specific serological method,which detect of antibodies withantigen coated beads.

-Immunoflourescent (IF)

-Western blot

-Polymerse chain reaction (PCR) analysis of DNA .Saliva,throat washing can be used to detect EBV

2.15**Treatment**

There is no treatment for an EBV infection resulting in mononucleosis except to treat symptoms.Acyclovir reduces EBV Shedding from the oropharynx administration, but it does not affect EBV-immortalized B cells. Acyclovir has no effect on the symptoms of mononucleosis and is of no proved benefit in the treatment of EBV –associated lympho-masin immunocompromised patients.(Geo *et al*.,2013)

2.16 Prevention

-Restricting intimate contact during acute mononucleosis can reduce the transmission of EBV but need lessly hamper contact with hamper contact with many person who are already seropositive.

The transmissibility of EBV during a symptomatic viral shedding remains problematic long after the acute illness.

-Avoidance of intimate contact is reasonable in the few instances when the complication of infection could devasting.

-Nosocomialtransmission of EBV is largely preventable by attention to precaution and hand washing. Isolation of person with acute infectious mononucleos isgenerally unnecessary(Garner and Simmons.,1983)

2.17.1 leukemia

Leukemiasare cancers of the white blood cells, which begin in the bone marrow. This information refers to four types of leukaemia; acute lymphocytic leukaemia, chronic lymphocytic leukaemia, acute myeloid leukaemia and chronic myeloid leukaemia.

Leukaemiasare grouped in two ways: the type of white blood cell affected – lymphoid or myeloid; and how quickly the disease develops and gets worse – acute leukaemia appears suddenly and grows quickly while chronic leukaemia appears gradually and develops slowly over months to years. (Hoffbrand*et al*.,2006)

2.18.1Types of leukemia

There are several types of Leukaemia. Some types appear suddenly and progress rapidly over days to weeks.

Leukemias are named according to the type of blood cells involved. The myeloid leukemias are those which involve the granulocytes red blood cells platelets and monocytes; the lymphocytic leukaemias are those which involve the lymphocytes.

2.18.2.1Acute lymphocytic leukemia (ALL)

Acute lymphocytic leukaemia is most common in children.its incidence is highest at 3-7 year,falling off by 10 years with asecondary rise after the of 40 (Hoffbrand*et al.,* 2006) .

2.18.2.2Chronic lymphocytic leukaemia (CLL)

This leukemia also affects the lymphocytes, but usually develops much more slowly than acute lymphocytic leukemia. It has peak incidence between 60and 80 year Hoffbrand*et al.,* 2006)

2.18.2.3Acute myeloid leukemia (AML)

This leukemia mainly affects adults, but can occur in children and adolescents. It is common form of acute leukemia in adults and increasing with age(Hoffbrand*et al*.,2006)

2.18.2.4Chronic myeloid leukemia (CML)

Chronic myeloid leukaemia isaclonal disorder of pluripotent stem cell. can occur at any age, but is uncommon below the age of 20 years. (Hoffbrand*et al* .,2006)

CHAPTER THREE

CHAPTER THREE

3.Materials and methods

3.1Study design

The study was ProspectiveCross sectional.

3.2Study period

This work carried outin the period between April to June 2015.

3.3Study area

This study was carried out-in Radiation Isotope Center-Khartoum(RICK)

3.4Study population

Patientswho had already diagnosedby haematologist as leukemic ones

3.5Sample size

A total of 80 subjects ofleukemic patients.

3.6Data collection

The data was collected direct oral interviewing questionnaire(questionnaire see in appendix(1)

3.7 Ethical consideration

An approval for the work was taken from Research Ethical Committee in Sudan university of Science and Technology,Leukemic patients were informed for the purpose of the study and its objectives.

3.8 Specimen collection and preservation

Five ml of venous blood was collected by using venous puncture technique the collected bloodwasdrawn into plain container,allowed to clot and then centrifuged at 3000 rpm for 5minutes. The sera were seprated intocryo tubes preserved at 2°C -8 °C until used.

3.9 Laboratory work

The preserved sera for leukemic patientsthen tested for antibodies raised against the viral capsid antigen (VCA) of EBV using ELISA.Figure 3.1show microtiter ELISA(See appendix-2)

3.10 Principle

The test kit contains micro titer strips with 8 break-off reagent wells coated with EBV-VCA.In the first reaction step,diluted samples are incubated in the wells.In the case of positive samples ,specificIgG antibodies will bind to antigens.To detect the bound antibodies ,a second incubation is carried out using an enzyme labeled anti-human IgG (enzyme conjugate) Catalysingacolour reaction.A third incubation is carried out using chromogen/substrate solution.The reaction is then stopped by adding acid .Colar intensity is determinedby micro ELISA reader see (Appendix-3) 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-6)

All reagentwere brought to room temperature approximately 30 minutes before used.

Washing buffer was prepared by adding 40ml of buffer to 360ml of distilled water in flask.

Samples were diluted by adding 10 µl to 1ml of samples diluents. 100 µl of calibrators, controls, and diluted sera were dispended into appropriate wells incubated for 30 min at room temperature (18°C to 25°C) and then washed three times using 300µl diluted washing buffer.Then 100 µl of enzyme conjugate (peroxide-labled anti-human IgG) were dispensed into all wells, incubated for 30 min at room temperature (18°C -25°C), and then washed three times using 300 m µl diluted washing buffer.

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

100 µl of stock solution (0.5Msulphuric acid) was added to the wells containingchromogen /substrate solution

The color intensity was read at wave length of 450nm within 30minutes of adding stop solution. Reading of results(see appendix-5)

3.12Calculation and interpretation of the result

Resultswere evaluated semi quantitatively by calculating a ratio of extinction value of the control or patient sample over the extinction value of calibrator 2.calculate the ratio according the following formula:

$$\frac{\text{Extinction value of the control patients sample}}{\text{Exctintion value of the calibrator2}} = \text{Ratio}$$

EURO IMMUN recommends interpreting results as follows:

Ratio <0.8: negative

Ratio ≥0.8to< 1.1: borderline

Ratio ≥1.1: positive

3.13 Data analysis

Data were analyzed by usingSPSSsoftware program version 21.0.Significance of differences was determine using chi-square test and statistical significance was set at p>0.05

CHAPTER FOUR

CHAPTER FOUR

4.Results

4.1 Detection of anti-EBV among the leukemic patients

The results demonstrated that 60/ 80(75.0%) were anti-EBVIgG positive and only 20(25.0%)were EBV negative among leukemic patient. (Table4.1)

Table4.1The seroprevalence of anti EBVamong theleukemic patients

Anti-EBV			
Study Group	Positive	Negative	Total
Leukemic Patients	60 (75.0%)	20(25.0%)	80(100%)

4.2 The effect of gender on EBV IgG prevalence

Table:4.2 revealed there was difference in the prevalence of EBV in males and females .The highest occurance of anti-EBV positive in males34(42.5%), females26(32.5%)

Table 4.2 The effect of gender on EBV Prevalence

Anti-EBVIgG			
Target Group	Positive	Negative	Total%
Males	34(42.5%)	15(18.8%)	49(61.3%)
Female	26(32.5%)	5 (6.3%)	31(38.8%)
Total	60(75.0%)	20(25%)	80(100%)

P=0.189

4.3The effect of age on EBV IgG prevalence

Table (4.3) showed that the age of leukemic patients prevalence of EBVin this study(1-20)year which represent21(26.3%)were among(21-40) 16(20%) wereamong(41-60) represent 19(23.8)and 4(4%)among(61-80)

Table 4.3 The effect of age on EBV Prevalence

Anti-EBV			
Age group	Positive	Negative	Total
(1-20)	21(26.3%)	9(11.3%)	30(37.5%)
(21-40)	16(20%)	4(5%)	20(25.0%)
41-60)	19(23.8%)	7(8.8%)	27(32.5%)
(61-80)	4(4.0%)	0(0.0%)	4 (4.0%)
Total	60(75%)	20(25.0%)	80(100%)

P=0.56

4.4 **The effect of type of leukemia on EBV IgG Prevalence**

The result in table (4.4) showed that the prevalence of Epstein-Barr virus(VCA).IgG antibodies within type of leukemia increasing of Acute lymphoid leukemia Acute lymphobastic leukemia 21(23%),Chronic Acute myeloid leukemia 8 (8.9%) WhileChronic myeloid leukemia 18(20%).Table (4.4)

Table4.4 The effect of type of leukemia on EBV Prevalence

Anti-EBVIgG			
Types of Leukemia	Positive	Negative	Total
ALL	21(23%)	5(5.6%)	26(28%)
CLL	13(14.4%)	5(5.6%)	18(20%)
CML	18(20%)	8(8.9%)	26(28.9%)
AML	8(8.9%)	2(2.2%)	10(11.1%)
Total	60(75.0%)	20(25%)	80(100%)

P=0.77

Key:

ALL:acute lymphoblastic leukemia AML: acute myeloid leukemia

CLL: chronic lymphocytic leukemia CML: chronic myeloid leukemia

CHAPTER FIVE

CHAPTER FIVE

5.Discussion

5.1 Discussion

EBV infections are quite prevalent, affecting more than 90% of individuals during the first two decades of life worldwide(In developing countries, primary infections occur mainly in young children and are often asymptomatic .In developed countries, primary EBV infections are manifested mainly as infectious mononucleosis, and affect adolescents and young adults (papeschand Watkins., 2001).

The aim of this study was determine that the prevalence of EBV infection in leukemic by detection of EBV-IgG antibodies by using the enzyme linked immunosorbentassay(ELISA).

Seroprevalence of EBV IgG(EBV) was found to be (75.0%) of leukemicpatients.

The distribution of anti-EBV positive in 80 cases was 21(23%),13(14%), 18(20%),8(8.9%),wereacute lymphoblastic leukemia,chronic lymphocytic leukemia, chronic myeloid leukemia and acute myeloid leukemia respectively.

These results showedthat EBV was higherprevelanceamong patients with acute lymphoblastic leukemia. This result agreed with (Hussain*etal*.,2011) 23(79%),5(17%),1(3.4) acute lymphoblastic leukemia, acute myeloid leukemia chronic myeloid leukemia, respectively. The currentresults was less percentage than the previous study.That may apparently due to large sample size.

The current results also agreed with(Elawad*et al*., 2015) the results showed 33/88 Acute lymphoblastic leukemia (ALL),28/40 Chronic myeloid leukemia,which found the evidence of the involvement of EBV in patients with leukemia. These study also agreed with(Sehgal *et al*; (2010).who examined 8/25 acute lymphoblastic leukemia(ALL) These

mentioned study indicate that asignificant number of patients with ALL show evidence of active EBV replication.Also the result showed that EBV higher in age groups between (1-20) year which represent 21(26.3%)agreed with (Hussain*et al*.,2011)

The resultsobtained demonstrated that there was higher increase of EBVamong males more than females, males34(42.5%),females 26(32.5%) this agreed with(Elwad*et al*.,2014)

5.2 Conclusion

Based on the results of this study suggested the evidence association between EBV and leukemia.

5.3 Recommendation

Increase the sample size to maximize the benefits for better full diagnosis of EBV.

Specific and sensitive EBV detection techniqueshould be used at early stage in leukemia like polymerase chain reaction(PCR)

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Referance

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APPENDICES

EUROIMMUN Medizinische Labordiagnostika AG

(EV_0010_F_DEUK_A04) 13351000-0092_EV_0010_F_DEUK_A04.dnc

ELISA-Inkubation
ELISA Incubation

		Antigen-beschichtete Reagenzgefäße *antigen-coated wells*	
	Pipettieren: 100 µl je Reagenzgefäß ***Pipette:*** *100 µl per well*	Kalibratoren, Kontrollen, verdünnte Proben *calibrators, controls, samples*	
1.	**Inkubieren:** 30 min bei Raumtemperatur (18°C bis 25°C) ***Incubate:*** *30 min at room temperature (18°C to 25°C)*		
	Waschen: 300 µl (man.)/450 µl (aut.) je Reagenzgefäß Einwirkzeit: 30-60 s je Waschzyklus ***Wash:*** *300 µl (man.)/450 µl (aut.) per well* *residence time: 30-60 s per washing cycle*	3x	
	Pipettieren: 100 µl je Reagenzgefäß **Pipette:** 100 µl per well	Enzymkonjugat *enzyme conjugate*	
2.	**Inkubieren:** 30 min bei Raumtemperatur (18°C bis 25°C) ***Incubate:*** *30 min at room temperature (18°C to 25°C)*		
	Waschen: 300 µl (man.)/450 µl (aut.) je Reagenzgefäß Einwirkzeit: 30-60 s je Waschzyklus ***Wash:*** *300 µl (man.)/450 µl (aut.) per well* *residence time: 30-60 s per washing cycle*	3x	
	Pipettieren: 100 µl je Reagenzgefäß **Pipette:** *100 µl per well*	Chromogen/Substrat *chromogen/substrat*	
3.	**Inkubieren:** 15 min bei Raumtemperatur (18°C- 25°C) ***Incubate:*** *15 min at room temperature (18°C- 25°C)*		
	Pipettieren: 100 µl je Reagenzgefäß **Pipette:** *100 µl per well*	Stopplösung *stop solution*	
	Auswerten: Photometrische Messung (450 nm) ***Evaluate:*** *photometric measurement (450 nm)*		

EUROIMMUN Medizinische Labordiagnostika AG

Qualitätskontrollzertifikat
Quality Control Certificate

Produkt: *Product:* **Anti-EBV-CA ELISA (IgG)**

Best.-Nr.: *Order No:* **EI 2791-9601 G**

Ch.-B.: *Lot:* **E150312AW**

Verw. bis: *Exp. Date:* **11-Mar-2016**

		Referenzwert *Reference value*		Valider Bereich *Valid range*	
Kalibrator 1 *Calibrator 1*	200 RU/ml	1,939	O.D.	> 0,700	O.D.
Kalibrator 2 *Calibrator 2*	20 RU/ml	0,315	O.D.	> 0,140	O.D.
Kalibrator 3 *Calibrator 3*	2 RU/ml	0,116	O.D.		
Pos. Kontrolle 1 *Pos. Control 1*	quantitativ *quantitative*	124	RU/ml	87 - 161	RU/ml
Pos. Kontrolle 1 *Pos. Control 1*	semiquantitativ *semiquantitative*	4,0	Ratio	2,2 - 5,8	Ratio
Neg. Kontrolle *Neg. Control*	quantitativ *quantitative*	2	RU/ml	0 - 15	RU/ml
Neg. Kontrolle *Neg. Control*	semiquantitativ *semiquantitative*	0,1	Ratio	0 - 0,7	Ratio

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

Die Charge wurde von der Qualitätskontrolle getestet und erfüllt alle Spezifikationen.
The lot has been tested by the quality control and fulfills all specifications.

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 are 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 **semiquantative 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: (1st)	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: (2nd)	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: (3rd)	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.

Pipetting protocol

	1	2	3	4	5	6	7	8	9	10	11	12
A	C 2	P 6	P 14	P 22			C 1	P 4	P 12	P 20		
B	pos.	P 7	P 15	P 23			C 2	P 5	P 13	P 21		
C	neg.	P 8	P 16	P 24			C 3	P 6	P 14	P 22		
D	P 1	P 9	P 17				pos.	P 7	P 15	P 23		
E	P 2	P 10	P 18				neg.	P 8	P 16	P 24		
F	P 3	P 11	P 19				P 1	P 9	P 17			
G	P 4	P 12	P 20				P 2	P 10	P 18			
H	P 5	P 13	P 21				P 3	P 11	P 19			

The pipetting protocol for microtiter strips 1-4 is an example for the semiquantitative analysis of 24 patient samples (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 the following formula:

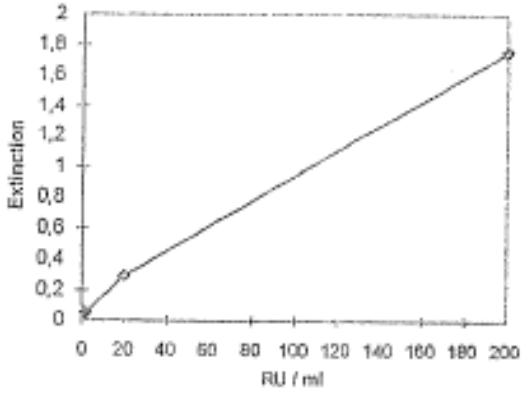
$$\frac{\text{Extinction of the control or patient sample}}{\text{Extinction of calibrator 2}} = \text{Ratio}$$

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 re-tested 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/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 R^2 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.

Antibodies against	n	Anti-EBV-CA ELISA (IgG)
Adenovirus	10	0%
Chlamydia pneumoniae	5	0%
CMV	3	0%
Influenza virus A	4	0%
Influenza virus B	9	0%
Measles virus	9	0%
Mumps virus	9	0%
Mycoplasma pneumoniae	3	0%
Parainfluenza virus Pool	10	0%
RSV	8	0%
Rubella virus	10	0%
VZV	5	0%

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.

Intra-assay variation, n = 20		
Serum	Mean value (RU/ml)	CV (%)
1	47	7.4
2	90	5.8
3	93	4.2

Inter-assay variation, n = 4 x 6		
Serum	Mean value (RU/ml)	CV (%)
1	47	8.2
2	90	3.2
3	93	5.4

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.

n = 111		INSTAND / Labquality		
		positiv	borderline	negativ
EUROIMMUN Anti-EBV-CA-ELISA (IgG)	positive	92	0	0
	borderline	3	1	0
	negative	0	0	15

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 exanthema. 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, eyes 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 titer 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** [15].

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 $CSQ_{path.-spec.}$ (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].

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Anti-EBV-CA ELISA (IgG)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG-CLASS	SUBSTRATE	FORMAT
EI 2791-9601 G	Epstein-Barr virus capsid antigen (EBV-CA)	IgG	Ag-coated microplate wells	96 x 01 (96)

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 an enzyme-labelled anti-human IgG (enzyme conjugate) catalysing a colour reaction.

Contents of the test kit:

Component	Colour	Format	Symbol
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/H_2O_2, 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	

LOT	Lot		Storage temperature
IVD	In vitro determination		Unopened usable until

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.