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Seroprevalence of Epstein- Barr Virus among Lymphoid Leukemia Patients in Khartoum State

الإنتشار المصلي لفيروس ابشتاين بار لدى المرضى المصابين بسرطان الدم الليمفاوي في ولاية الخرطوم

A dissertation submitted in partial fulfillment for the requirements of MSc in Medical Laboratory Science (Microbiology)

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الآية

قال الله تعالى:

{يَرْفَعِ اللّهُ الّذِينَ آمَنُوا مِنكُمْ وَالّذِينَ أُوتُوا الْعِلْمَ دَرَجَاتٍ}

صدق الله العظيم

سورة المجادلة: الآية (11)

DEDICATION

I dedicate this work to

My mother

and father

ACKNOWLEDGEMENT

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

I would like to thank my supervisor **Prof. Humodi Ahmed Saee**d for his invaluable advice and guidance.

I owe deep gratitude to several persons for their substantial help namely Mohammed Abu Baker ELtaib, Mohammed Kararr, Mohammed Salah and Sayed Mutasim.

ABSTRACT

Epstein-Barr virus (EBV) prevails among more than 90% of the adult population worldwide. The objective of this study was to determine seroprevalence of EBV among lymphoid leukemia patients in Khartoum State.

A total of 86 patients were selected from Radiation and Isotope Center of Khartoum (RICK) in the period between February and September 2016. Socioeconomic data such as age, gender, type of leukemia and disease stage were collected from patients by questionnaire. Blood specimen was collected from each patient in ***Ethylenediaminetetra-Acetic Acid*** (EDTA) container. Plasma was obtained by centrifugation of the blood at 3000 rpm for 5minutes. Enzyme linked immunosorbent assay (ELISA) was used to detect Epstein- Barr virus EBV IgG antibody.

The results revealed that the prevalence of EBV among lymphoid leukemia patients was 81 (94.2%). 56 (65.1%) in males and 25 (29.1%) in females. High prevalence was reported among age group (61-80) 14 (100%). EBV IgG prevalence 60 (93.7%) was detected among patients with acute lymphoid leukemia (ALL) and 21 (95.4%) in chronic lymphoid leukemia patients (CLL). According to disease stage the prevalence of EBV IgG among ALL patients in different stages was follows; 36 (97.2%) in Pre B cell stage, 19 (86.3%) in B cell stage and 6 (100%) in T cell stage while the prevalence of EBV IgG among CLL patients was showed as follows; 5 (100%) in stage I, 8 (88.8%) in stage II and 8 (100%) in stage III.

It is concluded that there is high prevalence of EBV among lymphoid leukemia patients. Further studies using large number of specimens and advanced technique were recommended to validate this study.

المستخلص

فيروس ابشتاين بار يسود في أكثر من 90% من سكان العالم البالغين، هدفت هذه الدراسة الى تحديد مدى انتشار فيروس ابشتاين بار بين مرضى سرطان الدم الليمفاوي في ولاية الخرطوم، تم اختيار 86 مريض من المركز القومي للعلاج بالأشعة و الطب النووي بولاية الخرطوم Radiation and Isotope Center of Khartoum في الفترة ما بين فبراير الي سبتمبر من العام 2016. تم جمع عينة دم من كل مريض في حاوية تحتوي مركب الاديتا (EDTA)، وتم استخلاص البلازما من دم الاديتا بواسطة الطرد المركزي على سرعة 3000 لمدة 5 دقائق, مقياسة الامتصاص المناعي المرتبط بالانزيم (اليزا ELISA) استخدمت للكشف عن الأجسام المضادة من النوع IgG لفيروس ابشتاين بار في عينات الدم.

و كشفت النتائج عن معدل انتشارعالي للأجسام المضادة IgG لفيروس ابشتاين بار بين مجموع عينات الدم التي أخذت من مرضى سرطان الدم الليمفاوي كانت 81 (94.2%). 56 (65.1%) عند الزكور و 25 (29.1%) عند الاناث, مع انتشار عالي في الفئة العمرية بين (61-80) سنة 14 (100%).

وكان انتشار الأجسام المضادة IgG المكتشف لفيروس ابشتاين بار 60 (93.7%) لدى مرضى سرطان الدم الليمفاوي، و21 (95.4%) لدى مرضى سرطان الدم الليمفاوي المزمن، و تبعا لطور المرض فقد وجد انتشار معدل الأجسام المضادة IgG لفيروس ابشتاين بار في مرضى سرطان الدم الليمفاوي الحاد في اطوار مختلفة كما يلي: 36 (97.2%) طور المرض Pre B cell، 19 (86.3%) في طور المرض B cell و 6 (100%) في طور المرض T cell؛ بينما انتشار معدل الأجسام المضادة IgG لفيروس ابشتاين بار في مرضى سرطان الدم الليمفاوي المزمن ظهرت كما يلي: 5 (100%) في طور المرض I، 8 (88.8%) في طور المرض II و 7 (100%) في طور المرض III.

و خلصت هذه الدراسة الى و جود انتشار عالي لفيروس ابيشتاين بار لدى مرضى سرطان الدم الليمفاوي. و أوصت باجراء مزيد من الدراسات و زيادة عدد العينات و استخدام طرق تشخيصية متقدمة للتأكد من صحة هذه الدراسة.

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ABBREVIATIONS

ALL: acute lymphoid leukemia.

BL: Burkitt's lymphoma.

CAEB: chronic active Epstein barr.

cHL: classic Hodgkin's lymphoma.

CLL: chronic lymphoid leukemia.

CTL: cytotoxic T- cell.

EA: early antigen.

EBER: Epstein barr RNA.

EBNA: Epstein barr nuclear antigen.

EBV: Epstein barr virus.

ELISA: enzyme linked immunosorbent assay.

GC: gastric carcinoma.

HH-4: human herpes- 4.

HL: Hodgkin's lymphoma.

HIV: human immune virus.

HTLV: human T- cell lymphotropic virus.

IEA: immediate early antigen.

IgA: immunoglobulin A.

IgG: immunoglobulin G.

IgM: immunoglobulin M.

IL- 10: interleukin- 10.

IM: infectious mononucleosis.

IR: internal repeat.

LCL: lymphoblastoid cell lines.

LCV: lymphocryptovirus.

LMPs: latent membrane proteins.

MS: multiple sclerosis.

NHL: non Hodgkin's lymphoma.

NPC: nasopharyngeal carcinoma.

ORF: open reading frame.

PTLD: post transplant lymphoproliferative disease.

RNA: ribonucleic acid.

TR: terminal repeat.

VCA: viral capsid antigen.

XLP: x- linked lymphoproliferative syndrome.

CHAPTER ONE

INTRODUCTION AND OBJECTIVES

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1.1.Introduction

Epstein-Barr virus (EBV) is named after the virologists (Epstein and Barr)who first observed it under electron microscope in cultures of lymphoblasts from Burkitt's lymphoma. The EBV, also called human herpesvirus 4 (HH-4), is a virus of the herpes family and one of the most common viruses in humans (Maeda*et al*., 2009).

Epstein-Barr virus (EBV) is a ubiquitous virus which belongs to the γ herpesvirus subfamily.1) γ herpesviruses are well-known as tumor viruses that express virus cancer genes and immortalize infected lymphocytes (Kimura *et al*., 2013).

The virus consists of a nucleocapsid containing a 184 kbp double stranded (ds) DNA molecule surrounded by 162 capsomers. The nucleocapsid is surrounded by a protein-rich tegument, which in turn is surrounded by an envelope. The genome of the virus is structured similar to other herpesviruses, in which there are unique long and short regions, separated by a long run of internal repeats, and flanked by terminal repeats. There are about 190 genes per genome (Tselis, 2013).

EBV has a world-wide distribution being able to establish a lifelong infection in more than 90% of individuals. Primary infection is usually asymptomatic and only when it is delayed until adolescence or adulthood a benign lymphoproliferative

disease, known as infectious mononucleosis (IM), may occur (Dolcetti and Carbone, 2010).

The presence of EBV genomes and constant expression of viral protein, strongly support the involvement of EBV in the pathogenesis of a wide Spectrum of human malignancies. These include lymphomas of B, T and NK cell origin such as the immunoblastic lymphoma of immune suppressed, endemic Burkitt's lymphoma (BL), Hodgkin's lymphoma (HL). But also carcinoma of the nasopharynx and leiomyosacoma arising in organ transplant patient and HIV infected individuals (Chen *et al*., 2007).

EBV can be considered as the prototype of oncogenic viruses that behave as direct transforming agents. In fact, in classical EBV-associated tumours, the virus genome is present in virtually all neoplastic cells, which show the expression of viral RNAs and proteins that variously contribute to the induction of the transformed phenotype. On the basis of these features and of the strict association with distinct tumour types, EBV has been classified as a group I carcinogen. An additional compelling factor is the presence of homogeneous (clonal) EBV episomes detected with the use of the virus termini assay in several EBV-related tumours (HL, NPC, BL) as well as in some pre-neoplastic lesions. Besides the well-defined group of tumours pathogenically associated with EBV according to the criteria mentioned above, the presence of this herpesvirus has been variably detected in a broad spectrum of other tumours for which a causal role of EBV seems unlikely. These tumours include also chronic lymphocytic leukemia.

Review available data suggesting a possible role of EBV and a direct or microenvironmental progression factor in a fraction of CLL (Dolcetti and Carbone, 2010).

1.2. Rationale

The cause of lymphoid leukemia is unknown and research in this field is being carried out all the time. However, some factors are thought to increase risk of it by exposure to radiation, benzene, and some genetic condition. Viruses have also been linked to some forms of lymphoid leukemia. For example, certain cases are associated with viral infection such as T-lymhpotropic virus (HTLV-1 and -2, causing T-cell leukemia/lymphoma). Results of several studies have suggested a probable etiologic association between Epstein-Barr virus (EBV) and lymphoid leukemia's; therefore, the aim of this study was to estimate seroprevalence of Epstein-Barr virus (EBV)among lymphoid leukemia patient by using serological methods like enzyme linked immunosorbent assay in Khartoum.

1.3. Objectives

1.3.1. General objective

To determine seroprevelance of Epstein-Barr virus (EBV) among lymphoid leukemia patients.

1.3.2. Specific objectives

A. To detect IgG (CA) antibody of Epstein-Barr virus (EBV) in plasma obtained from lymphoid leukemia patients.

B. To determine possible involvement of Epstein- Barr virus in the etiology of lymphoid leukemia.

CHAPTER TWO

LITERATURE REVIE

CHAPTER TWO

LITERATURE REVIEW

2.1. Historical background

Epstein- Barr virus (EBV) was first identified in London in 1964 by Anthony Epstein and Yvonne Barr. Showed viral particles with typical herpes virus morphology in malignant cells by using electron microscopy of cell culture derived from tumor biopsies of Ugandan children affected by Burkitt's lymphoma (Williams and Crawfordd, 2006).

In 1967, a technician in the Henle laboratory developed a febrile illness with pharyngitis, abnormal peripheral blood lymphocytes, and a positive heterophile antibody test (known as Paul and Bunnel's test), consistent with a diagnosis of infectious mononucleosis (IM), a self-limiting B cell lymphoproliferative disease. When she went back to work, she was found to have developed antibodies against the "EB" virus, providing the first clue that EBV might be the causative agent of IM (Gru *et al*., 2015).

Serological studies in a large cohort of Yale college students and in healthy populations later confirmed that EBV is in fact the cause of IM and revealed that EBV has ubiquitous distribution worldwide, including areas endemic for Burkitt's lymphoma, where uniquely high titers of antibodies against the virus were detected (Gru *et al*., 2015).

In 1973 was observed early on that EBV-infected cells from IM patients grew spontaneously in vitro, experimentally proved that EBV can efficiently infect and immortalize quiescent human B cells (but not T cells), producing continuous B cell lymphoblastoid cell lines (LCL), which express many EBV-encoded proteins, display several features of "transformed cells," and after prolonged culture develop an aneuploid karyotype and produce transplantable tumors in mice. LCL could also be easily established when peripheral blood mononuclear cells (PBMC) from EBV-seropositive individuals were cultured in vitro. Later work showed that when EBV "seropositive" PBMC are transplanted into immunosuppressed SCID mice (hu-PBL-SCID), they produce aggressive EBV-positive B cell lymphomas that resemble human post-transplant lymphoproliferative disorders (PTLD), with a frequency that is affected by the cytokine microenvironment and by the genetic background of the EBV-seropositive donor (Gru *et al*., 2015).

2.2. Classification

Epstein-Barr virus (EBV) is a member of the herpesvirus family. As with other herpesviruses, EBV is an enveloped Latency virus that contains a DNA core surrounded by an icosahedral nucleocapsid and a tegument. Family members include herpes simplex I and II and varicella zoster virus (α -herpes-virus subfamily), cytomegalovirus and human herpes virus (HHV)-6 and HHV-7 (β -herpes virus subfamily), and HHV-8 and EBV (γ-herpes virus subfamily) (Carbone *et al*., 2008).

Epstein-Barr virus (EBV) is also classifying as lymphocryptovirus (LCV), lymphocryptovirus (LCV) genomes are very similar in structure and organization. Structural features that are shared between LCV members include 0.5kbp terminal repeats (TR), 3 kbp internal repeats (IR1) and short internal repeats (IR2, IR4). The open reading frames (ORFs) encode co-linearly homologous, antigenically-related, structural and non-structural proteins (Riviller *et al*., 2002).

2.3. Type and strains of Epstein-Barr virus (EBV)

EBV isolates worldwide can be grouped into Type 1and Type 2, aclassification based on the EBNA2 gene sequence.

Type 1 EBV is the most prevalent worldwidebut Type 2 is common inparts of Africa.

Type 1 transforms human B cells into lymphoblastoid cell lines much more efficiently than Type 2 EBV (Tzellos and Farrell, 2012).

2.4. Structure

The virus consists of a nucleocapsid containing a 184 kbp double stranded (ds) DNA molecule surrounded by 162 capsomers. The nucleocapsid is surrounded by a protein-rich tegument, which in turn is surrounded by an envelope. The genome of the virus is structured similar to other herpesviruses, in which there are unique long and short regions, separated by a long run of internal repeats, and flanked by terminal repeats. There are about 190 genes per genome (Tselis, 2013).

The nomenclature for EBV open-reading frames (ORFs) is based on the BamHI-restriction fragment in which they are found. For example, the BARF1 ORF is found in the BamHI A fragment, and extends rightwards (Young and Rickinsons, 2004).

The many EBV ORFs are divided into latent and lytic genes (further divided into immediate early genes, early genes, and late genes). Most of these genes are translated into proteins and several lytic genes encode for human homologues (Young and Rickinsons, 2004).

In addition, some latent genes are non-translated; this is the case for EBV-encoded RNA (EBER)-1 and -2 (Young and Rickinsons, 2004).

EBV also encodes at least 17 micro-RNAs, arranged in two clusters: ten are located in the introns of the viral BART gene, and three adjacent to BHRF1 (Cai *et al.*, 2006).

The viral genome also contains a series of 0.5-kb terminal direct repeats at either end and internal repeat sequences that serve to divide the genome into short and long unique sequence domains that have most of the coding capacity (Cai *et al.*, 2006).

These terminal repeats are good markers to determine if EBV-infected cells are from the same progenitor: when EBV infects a cell, the viral DNA circularizes and mainly persists as a circular episome with a characteristic number of terminal repeats that depends on the number of terminal repeats in the parental genome, with some variation introduced during viral replication. If the infection is

permissive for latent infection but not replication, future generations will have EBV episomes with the same number of terminal repeats (Cai *et al*., 2006).

2.5. Viral antigens

Epstein-Barr virus (EBV) like other herpesvirus has productive lytic phase and latent phase.

During the lytic cycle, has a regulatory proteins belonging to the immediately early antigen (IEA) and early antigen (EA) groups are synthesized to allow the production of viral DNA (EBV-DNA), the virion structural proteins (viral capsid antigen, VCA) and membrane proteins (MA) (De Paschale and Clerici, 2012).

During the latent phase, EBV nuclear antigens (EBNAs) and three latent membrane proteins (LMPs) are expressed in infected cells. EBNAs represent of at least six proteins (EBNA 1-6). EBNA-1 is responsible for maintaining the episomal state of EBV DNA in infected cells, and EBNA-2 seems to be involved in the immortalization of B lymphocytes (De Paschale and Clerici, 2012).

The LMPs (LMP1, LMP2A and LMP2B) may also play a role in the process of immortalization, and oncoprotein LMP-1 appears to be responsible for most of the effects altering the growth of infected B cells (Paschale and Clerici, 2012).

Depending on the expressed antigens, it is possible to distinguish four types of latency, each of which is typical of the diseases associated with EBV, As EBNA-3 is a target of CD8+ lymphocytes (Hislop *et al*, 2007), latent cells are normally eliminated by cytotoxic T lymphocytes in immunocompetent patients, whereas the

transformed cells can proliferate and cause various lymphoproliferative disorders in Immunosuppressed patients (De Paschale and Clerici, 2012).

2.6. EBV products and patterns of EBV gene expression

The EBV genome encodes a series of products interacting with or exhibiting homology to a wide variety of antiapoptotic molecules, cytokines, and signal transducers, hence promoting EBV infection, immortalization, and transformation (Carbone *et al*., 2008).

Based on patterns of expression of the EBV genome, three types of latent gene expression have been described:

Latency I, II, and III, during latency I, Epstein–Barr nuclear antigen 1 (EBNA-1) and the two small non-coding Epstein–Barr RNAs (EBERs) are expressed (Carbone *et al*., 2008).

EBV gene expression in latency II is usually limited to EBNA-1, the EBERs, latent membrane protein (LMP)-1 and LMP-2A and LMP-2B (Carbone *et al*., 2008).

Latency III usually involves the unrestricted expression of all EBNAs, EBERs,and LMPs (Carbone *et al*., 2008).

Latency I is generally associated with the EBV-related Burkitt's lymphoma, latency II has been associated with classic Hodgkin's lymphoma (cHL) and T-cell non-Hodgkin's lymphoma (NHL), and latency III occurs mainly in immunocompromised individuals suffering from post-transplant

lymphoproliferative disorders (PTLDs) and HIV-associated lymphoproliferative disorders and in lymphoblastoid cell lines (Carbone *et al*., 2008).

Furthermore, EBV encodes the following important proteins that show sequence and functional homology to diverse human proteins: BCRF-1 and interleukin (IL)-10, BHRF-1 and BCL-2, BARF-1 and intracellular adhesionmolecule 1 (Carbone *et al*., 2008).

2.7. Replication

EBV as a lymphocryptovirus, it possesses the unique ability to infect, activate and clonally expand B lymphocytes, and then persist as a latent infection within these cells. During primary infection, EBV, transmitted via saliva, enters naive B cells in the tonsil by attaching its surface glycoprotein gp350 to complement receptor 2 (CD21) on the surface of mature B cells and follicular dendritic cells. Immediately following initial B-cell infection, EBV expresses two homologues of the cellular anti-apoptotic Bcl-2 protein (BALF1 and BHRF1), which are essential for the survival of newly infected cells. EBV drives the infected B cell out of the resting state to become an activated B blast and then takes advantage of the normal pathways of B-cell differentiation to enable the B blast to become a latently infected resting memory B cell. To achieve this, the virus uses a series of different latency transcription programmes. After entering naive B cells, EBV first employs the latency III or 'growth' programme to express all viral latent proteins, namely the Epstein–Barr nuclear antigens (EBNAs) 1, 2, 3A, 3B, 3C and LP, and the

latent membrane proteins (LMP) 1, 2A and 2B. The thereby activated B blast enters a tonsillar germinal centre where it downregulates the expression of the EBNA proteins 2, 3A, 3B, 3C and LP. Ongoing expression of EBNA1, LMP1 and LMP2 (latency II or 'default' programme) enables the infected B cell to proceed through a germinal centre reaction to become a memory B cell. The EBV-infected memory B cell exits from the germinal centre and circulates in the blood; it expresses no viral proteins except during cell mitosis, when it expresses only EBNA1 (latency I). EBNA1 engages the host cell DNA polymerase, thereby enabling duplication of each EBV genome and transmission of the genome to each daughter cell. The absence of viral protein expression allows the virus to persist as a latent infection in memory B cells despite a healthy immune response. When latently infected memory B cells return to the tonsils, they can terminally differentiate into plasma cells, which initiate the lytic (replicative) transcription programme with the production of infectious virus. The released virions infect tonsil epithelial cells where the virus rapidly replicates and sheds continuously into saliva so that it can be transmitted to new hosts. Newly formed virus can also infect additional naive B cells in the same host (Pender and Burrows, 2014).

2.8. Transmission of infection

EBV transmission takes place through oropharyngeal secretion. In adolescent and adult cases of infectious mononucleosis, intimate kissing has been the main route of transmission whereas saliva (Gequelin *et al.*, 2011).

EBV infection frequently takes place among smaller children of low socioeconomic status, in nurseries and when sharing a room (Crawford *et al*., 2002).

EBV has been detected in cervical secretions 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 a major risk factor for post-transplant lymphoproliferative disease (PTLD) (Scheenstra *et al*., 2004).

2.9. Epidemiology

EBV infections are quite prevalent, affecting more than 90% of individuals during the first two decades of life worldwide (Maeda *et al*., 2009).

In developing countries, infection occurs early in life, and most early childhood infections are subclinical(Prabhu and Wilson, 2016).

When primary infection is delayed until later childhood or adolescence, it manifests in approximately 25–75% of cases as infectious mononucleosis (Prabhu and Wilson, 2016).

Epstein–Barr virus (EBV) is a human cancer-associated virus that infects >90% of the global population (Cohen *et al*., 2011).

For the past two decades, increasing interest has focused on the EBV-associated epithelial cancers that represent 80% of all EBV-associated malignancies (Cohen *et al*., 2011).

Among these, nasopharyngeal carcinoma (NPC) and EBV-associated gastric cancers (EBVaGCs) is the most common, with 78 000 and 84 000 new cases, respectively, reported annually worldwide (Cohen *et al*., 2011).

The incidence and mortality rates of NPC are remarkably high in southern China and South-East Asia, but NPC is rarely seen in Western countries (Cohen *et al*., 2011).

Burkitt's lymphoma has three clinical variants are recognized; endemic, sporadic, and immunodeficiency-associated (Kimura *et al*., 2013).

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, and this type the association to EBV is nearly 100% (Kimura *et al*., 2013).

Sporadic Burkitt's lymphoma, which is seen in children and young adults throughout the world, only has a 30% association to EBV (Kimura *et al*., 2013).

Immunodeficiency-associated Burkitt's lymphoma is primarily seen in association with the human immunodeficiency virus infection (Kimura *et al*., 2013).

2.10. Pathogenesis

EBV infects B cells and establishes a life-long infection; the so-called latent infection. In the primary infection, cell-free EBV in the saliva infects naïve B cells in the oropharynx (Thorley-Lawson and Gross, 2004).

EBV attaches to the cell surface protein CD21, the primary EBV receptor through the viral envelope glycoprotein gp350/220 (Kimura *et al.*, 2013). For the penetration of the virus into cell membrane, the viral glycoprotein complex gH-gL-gp42 and co-receptor HLA class II are necessary (Kimura *et al.*, 2013). EBV initiates a growth-transforming infection, causing naïve B cells to transform into proliferating blasts. In immunocompetent hosts, both EBV-specific cytotoxic T lymphocytes (CTL) and NK cells control the outgrowth of EBV-transformed cells during primary infection (Kimura *et al.*, 2013). Primary EBV infection is usually asymptomatic, but occasionally progresses to infectious mononucleosis, which resolves spontaneously after the emergence of EBV-specific immunity (Kimura *et al.*, 2013). EBV then establishes a latent infection in memory B cells. After convalescence, EBV persists latently in these memory B cells in an episomal form. These virus-infected cells persist at a low level for the entire lifetime (Thorley-Lawson and Gross, 2004). Occasionally, EBV-infected memory B cells differentiate into plasma cells that undergo lytic infection and produce viruses. Newly infected naïve B cells are controlled by CTL unless immunity is suppressed. In immunocompromised hosts, transformed cells become proliferating

blasts that can result in symptomatic disease, such as post-transplant lymphoproliferative disorders (Cohen *et al*., 2011).

2.11. Clinical significance

2.11.1. Infectious mononucleosis (IM)

IM is usually a benign, self-limiting lymphoproliferative disease most common in the Western world, which occurs in between 25% and 70% of young adults following primary EBV infection. Symptoms of IM appear around a month after primary infection and range from benign (lymphadenopathy, sore throat, fever and fatigue) to severe (fulminant hepatitis, liver necrosis). The appearance and increase of EBV-specific T lymphocytes leads to a gradual decline in symptoms (Okano and Gross, 2012). Rarely, severe or fatal IM develops if no successful EBV-CTL (cytotoxic T lymphocyte) response is mounted, which may be sporadic or linked to genetic disorders such XLP (X-linked lymphoproliferative syndrome (Okano and Gross, 2012). Certain cancers and other chronic conditions are epidemiologically linked to IM (Hjalgrim, 2012), with independent epidemiological links between HL (Hodgkin's lymphoma) and MS (Multiple sclerosis) following a clinical diagnosis of IM.

2.11.2. Lymphomas in Immunosuppressed Individuals

There exist several distinct classes of EBV-associated lymphoproliferative disorders in immunocompromised 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-related lymphoproliferative disorders. The most common gene-expression pattern in these disorders is latency III (Carbone *et al*., 2008).

2.11.3. Post-transplant lymphoproliferative disease (PTLDs)

McKhann CF and Penn I in 1969 has been well established that there is a higher incidence of lymphoproliferative disorders in transplant recipients of both a solid organ and bone marrow. According to the World Health Organization (WHO) classification, PTLDs may be classified into: (a) early lesions generally represented by EBV-driven polyclonal lymphoproliferations and (b) true monoclonal diseases, including polymorphic PTLD and monomorphic PTLD; the latter is further distinguished into Burkitt's lymphoma/Burkitt's-like lymphoma, diffuse large B-cell lymphoma (DLBCL), and cHL(Carbone *et al*., 2008).

2.11.4. HIV-Associated Lymphoproliferative Disorders

HIV-associated lymphoproliferative disorders are a heterogeneous group of diseases that arise in the presence of HIV-associated immunosuppression, a state that permits the unchecked proliferation of EBV-infected lymphocytes. Traditionally, these aggressive disorders include both central nervous system and systemic lymphomas. PEL also occurs and often involves EBV in addition to HHV-8 (Carbone *et al*., 2008).

2.11.5.Burkitt's lymphoma

Burkitt's lymphoma is endemic in many parts of Africa; it occurs sporadically all over the world. Two forms have been recognized: the endemic or African form commonly found in Africa and Papua New Guinea and the sporadic form found in North America, northern and Eastern Europe and the Far East (Prabhu and Wilson, 2016). A third form of Burkitt's lymphoma, called immunodeficiency-associated Burkitt's lymphoma, has also been reported particularly in HIV patients and allograft recipients with primary immunodeficiency (Gong*et al*., 2003).

Burkitt's lymphoma lesions can locally infiltrate surrounding tissues and may spread via the lymphatic system or blood vessels (Prabhu and Wilson, 2016).

Burkitt's lymphoma invariably originates in B cells. Microscopically, it is characterized by the presence of undifferentiated lymph reticular cells,which are highly proliferative and frequently show mitotic figures. Macrophages with abundant clear cytoplasm containing Burkitt's lymphoma cells or cell debris are scattered among the tumor cells, giving the characteristic "starry sky" appearance to histologic preparations (Prabhu and Wilson, 2016). Precise diagnosis is based on histologic, immunophenotypic and genetic features, and these remain considerations in planning appropriate therapy (Ferry, 2006).

2.11.6. Nasopharyngeal carcinoma (NPC)

NPC is a distinctive histological subtype of head and neck cancer arising from the nasopharynx. The incidence and mortality rates of NPC are remarkably high in

southern China and South-East Asia, but NPC is rarely seen in Western countries (LO *et al.,* 2004*).* According to the recent World Health Organization (WHO) classification, NPC is classified into two major histological subtypes: non-keratinizing carcinoma (either differentiated or undifferentiated) and keratinizing squamous cell carcinoma. Non-keratinizing NPC is consistently associated with EBV infection and accounts for the majority of NPCs in endemic regions. It is commonly described as lymphoepithelioma of the nasopharynx because of its prominent lymphocytic infiltration. EBV latent infection is also seen in keratinizing NPCs from endemic regions, but not in non-endemic regions. In summary, almost 98% of all NPCs are EBV-associated (Tsao *et al*., 2015).

2.11.7. Gastric carcinoma (GC)

EBV is found in 10% of GC, a malignant tumor of the stomach epithelium (Takada, 2000). EBV-positive GC is more prevalent in men than in women. Both the EBV-positive and negative forms of GC have a marked geographical distribution, being highly prevalent in Japan and the Andes. High EBV antibody titers are seen in GC cases. Given the high prevalence of EBV-positive GC, very little is known about whether differences exist in the genetic risk of developing EBV-positive versus EBV-negative GC and whether they have different epidemiological risk profiles. Many genes have been found to be somatically mutated in EBV-positive GC, but these mutations were not found in non-tumor tissue samples (Iizasa *et al*., 2012).

2.11.8. Hodgkin's lymphoma (HL)

Hodgkin's lymphoma (HL), also known as Hodgkin's disease, is a malignant neoplasm that develops from B lymphocytes of the germinal centers in lymph nodes in over 98% of all cases; in rare instances, it develops from post-thymic T cells (Campo *et al*., 2011). Not all HLs are EBV-positive, and the percentage of lymphomas positive for the virus varies by HL subtype, with EBV found in 50–80% of mixed-cellularity type HL (Okano and Gross, 2012). The EBV-positive HL is more common than EBV-negative HL in young children and older adults, with the opposite pattern in adolescents (Hjalgrim, 2012). EBV is also epidemiologically linked with HL, with a relative risk of HL following IM (Hjalgrim, 2012). In developed countries, the incidence rate of HL increases with age and occurs primarily in children of lower socioeconomic classes, perhaps because of early exposure to EBV. Genetics and an association with environmental agents, such as occupational wood working, radiation therapy, chemotherapy, long-term use of phenytoin and EBV infection, have been reported to play a role in the development of HL. Hodgkin's lymphoma is distinguished morphologically by the presence of malignant cells, called Reed-Sternberg cells, amid a background of non-neoplastic cells (Prabhu and Wilson, 2016).

2.11.9. Chronic Epstein–Barr virus infection of T and NK cells

EBV is primarily B lymphotropic, but in the context of immune suppression or genetic susceptibility, infection of T and NK cells may occur. One such condition is chronic active Epstein–Barr virus CAEBV. When primary EBV infection the symptoms do not spontaneously resolve in otherwise immunocompetent individuals, with infection of T and NK cells, recurrent or chronic bouts of IM-like illness result in abnormal patterns of EBV antibodies. Chronic active Epstein–Barr virus CAEBV patients have very high blood EBV loads (Okano and Gross, 2012). Chronic active Epstein–Barr virus CAEBV is more prevalent in south Asia, and particularly Japan, suggesting a particular host genetic background common in this region may also be a risk factor for chronic active Epstein–Barr virus CAEBV development (Kimura, 2005). Mutations in the *PRF1* gene were found in one patient who died following the onset of chronic active Epstein–Barr virus CAEBV and this disorder is increasingly treated by HCST (Kimura *et al*., 2001).

2.12. Laboratory diagnosis

Various laboratory tests have been used to diagnose EBV infection. In addition to tests for other diagnostically useful parameters (leukocytosis, lymphocytosis with atypical lymphocytes, abnormal liver function test, etc.), there are tests for detecting non-specific heterophile antibodies and specific anti-EBV

antibodies, as well as molecular biology methods used to detect EBV DNA (De Paschale and Clerici, 2012).

2.12.1. Specimens

These include lymphoid tissues, nasopharyngeal carcinoma tissue and saliva (for direct detection of viral antigens or EBV genomes), and blood (for serological tests) and peripheral blood (for blood smear) (Bhatia and Ichhpujani, 2008).

2.12.2. Direct antigen detection

Direct immunofluorescent antibody using specific viral antibodies is used to detect EBV in clinical specimens (Parija,2009).

2.12.3. Serodiagnosis

EBV infection stimulates production of a wide range of antibodies including the heterophilic antibodies. These heterophilic antibodies are IgM antibodies that recognize Paul-Bunnell antigen on sheep, horse, and bovine RBCs. Therefore, a serum form patient with acute mononucleosis agglutinates sheep, horse, or bovine erythrocytes (Parija, 2009).

2.12.3.1.Heterophile antibody test

Paul-Bunnell test: Paul-Bunnell test was first described by Paul and Bunnell for demonstration of heterophile antibodies in patients with infectious mononucleosis in 1932 (Parija S, 2009). This heterophile antibody, which is an IgM antibody, is not directed against EBV or EBV- infected cells, but is produced due to polyclonal

activation of B cells, by EBV. Such heterophilic are also found in serum sickness during drug reactivation and naturally occurring antibodies to the Forssman antigen. These antibodies are absent or present in a very low titer in serum of healthy individuals. The human blood to be tested is first absorbed by guinea pig kidney and then it is tested for agglutination activities that are directed against horse, sheep, or cow erythrocytes. The serum is activated at 56C for 30 minutes, and in doubling dilution is mixed with equal volume of a 1% suspension of sheep RBCs. The test is incubated at 37C for 4hours and is examined for agglutination. A serum titer of 100 or greater is considered a positive test and is suggestive of infectious mononucleosis. Differential absorption of agglutination with guinea pig and cow red cells is necessary for confirmation of diagnosis. Cow RBCs a absorb infectious mononucleosis heterophile antibodies from serum but not Forssman antibodies. Guinea pig kidney cells absorb Frossman antibodies, but not the infection mononucleosis heterophile antibodies. Both guinea pig kidney cells and bovine RBCs absorb the antibodies produced in serum sickness. A serum titer of 1:100 after adsorption with guinea pig cells is considered positive and is suggestive of acute infection mononucleosis. The heterophile antibodies are present in serum of 40-60% of patients with infectious mononucleosis in the 1st week of illness and in 80-90% of cases by 3rd or 4th week. The heterophile antibodies are usually present for 3 month but may persist for even as long as year (Parija, 2009).

2.12.3.2. Monospot test

Monospot test is a rapid slide agglutination test, which uses either horse RBCs or bovine RBCs. Bovine RBCs are more specific for acute infectious mononucleosis heterophile antibodies; hence they do not require differential absorption. Monospot test shows a specificity of 63-80%. It may show rare false positive with lymphoma or hepatitis (Parija, 2009).

2.12.3.3. Virus specific serological test

EBV infection is characterized by development of specific antibodies against different viral antigens, which appear during different stages of infection. Indirect immunofluorescence test, ELISA and Western blot are frequently used methods to detect antibodies against these antigens (Parija,2009). EBV specific antibody tests to detect specific antibody to viral antigens: Viral capsid antigen (VCA), early antigen (EA) and Epstein Barr nuclear antigen (EBNA) are useful confirmatory tests in diagnosis.

(i)Viral capsid antigen (VCA) antibody test: positive IgM antibody indicates recent EBV infection, while positive IgA is associated with nasopharyngeal carcinoma.

Demonstration of IgM VCA antibodies as well as antibodies against early antigens (EAs) in the serum indicates current infection with EBV. Demonstration of IgA VCN antibodies indicate past infection.

(ii)EB antibody test (for early antigens) is positive in Burkitt’s lymphoma and nasopharyngeal carcinoma.

(iii)EBNA antibody: A positive test indicates past infection (Chak, 2003).

2.12.4. Isolation of the virus

EBV can be isolated from saliva, peripheral blood, or lymphoid tissue by transformation of normal human lymphocytes obtained from umbilical blood. However, the procedure is tedious and time-consuming, hence seldom used. Attempt has also been made to culture spontaneously transformed B lymphocytes from EBV DNA or virus-infected patients (Parija,2009).

EBV can grow in Human Embryonic Lung Fibroblast culture. The cytopathogenic effect (CPE) produced by the virus is focal and is associated with refractile ballooning of the cells. The CPE unlike other viruses takes 2 or weeks to develop (Chak, 2003; Parija,2009)

2.12.5. Molecular diagnosis

DNA probe for Epstein-Barr early antigens has been used to detect and identify the EBV mRNA in the nuclei of EBV-infected lymphoid cells. PCR has been developed to detect EBV DNA in plasma during acute infectious mononucleosis. This EBV DNA levels decline during convalescence. They are not demonstrated in latency infected individuals (Parija,2009).

2.12.6. Other test

Blood count: Total leucocytes count ranges from 10,000 to 90,000cumm of blood, with a predominance of abnormal lymphocytes, B-cells which are diagnostically important. They constitute about 30-90% of total leucocytes and look like Monoblast /lymphoblast (Chak, 2003).

2.13. Treatment

There is no treatment for an EBV infection resulting in mononucleosisexcept to treat symptoms. Acyclovir reduces EBV Shedding from theoropharynx 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 lymphoma as in immunocompromised patients (Geo *et al.*, 2013).

2.14. Prevention

Vaccination of healthy EBV-seronegative young adults with recombinant gp350 prevents the infection by EBV, although it does not prevent asymptomatic infection. After vaccination, there was seroconversion to anti-gp350 antibodies persisting for 418 months and accounting for the protective effect, given that anti-gp350 antibody neutralizes EBV infectivity. Vaccination of rhesus monkeys with soluble rhesus lymphocryptovirus gp350 not only protects against infection but also reduces viral loads in animals that become infected with virus after challenge (Pender and Burrows, 2014).

2.15. Acute Lymphoblastic leukemia:

Acute lymphoblastic leukemia (ALL) is a malignant disease of the lymphopoietic system that is manifested by the slow but uncontrolled growth of abnormal, poorly differentiated lymphoid cells whose DNA synthesis time is significantly longer

than in normal tissues. These abnormal lymphoid cells can be found in the bone marrow, spleen and lymph nodes. Normal bone marrow elements usually are replaced or displaced by the abnormal cells. Acute lymphoblastic leukemia is predominantly a disease of children, although improved immunologic and cyto-chemical methods of identification have increased the frequency of diagnosis in adults. Acute lymphoblastic leukemia is the most common malignant disease in children and occurs most common frequently between the age of 2 and 10 years (Hussain *et al*., 2012).

2.16. Chronic lymphoid leukemia

Chronic lymphocytic leukemia (CLL) is the most common type of adult leukemia in the United States and Western Europe. CLL cells are small lymphoid B cells with scant cytoplasm having a regular outline. Nuclei contain clumped chromatin and nucleoli are usually absent. On bone marrow and peripheral blood smears the CLL variant with increased prolymphocytes (CLL/PLL), consists of more than 10%, but less than 55% prolymphocytes. Bone marrow histologic pattern may be nodular, interstitial, diffuse, or a combination of the three. These patterns correlate with prognosis. CLL cells express surface IgM or IgM and IgD, CD5, CD19, and CD23. Ig genes are rearranged. 40-50% of cases are un-mutated and 50-60% show somatic hypermutations. There is a group of genes that distinguishes the two genetic subytpes. ZAP-70 is among the genes whose expression is associated with an IgHV un-mutated CLL genotype. About 50% of CLL show 13q deletion, about

20% trisomy 12 and, less commonly, other genomic abnormalities. Low stage patients with mutated CLL have a better prognosis than those with un-mutated CLL. ZAP-70 and CD38 expression of are associated with a poor prognosis (Dolcetti and Carbone, 2010).

A small fraction (approximately 2-8%) of patients with CLL develops diffuse large B-cell lymphoma (DLBCL). This event has been termed Richter's syndrome or Richter's transformation and is associated with poor response to treatment and low survival times.Richter's syndrome is histologically characterized by confluent sheets of large cells that may resemble centroblast- or immunoblast-like cells. The majority of the reported DLBCL occurring in patients with CLL are clonally related to the previous CLL (Mao *et al*., 2007).An interesting finding is the occurrence of scattered Hodgkin and Reed Sternberg (HRS)-like cells in the background. In contrast to true Hodgkin lymphoma arising in CLL patients, the reactive typical background is absent and the HRS-like cells are surrounded by neoplastic B-cells. These HRS-like cells show evidence of EBV infection (Dolcetti and Carbone, 2010).

2.17. Previous studies

Study conduct to investigate incidence of EBV in pediatric leukemia in Sudan, the study aimed to investigate the association of EBV in childhood leukemia, the study shows EBV LMP1 gene transcripts were found in 29 (36.3%) of the 80

patients with leukemia but in none of the healthy controls ($P < .0001$). Of the 29 EBV^+ cases, 23 (79.3%), 5 (17.3%), and 1 (3.4%) were acute lymphoblastic leukemia, acute myeloid leukemia, and chronic myeloid leukemia, respectively (Hussain *et al*., 2012).

Study conduct to detect of Epstein Barr Virus IgG Antibodies among Leukemic Patients in Khartoum State, the study examined 80 subjects of leukemic patients; the results showed that the prevalence of Epstein-Barr virus infection was 80% (21/26) and 72% (13/18) among patients with CLL (AbdELraheem, 2015).

Study carried out in India to describe the results related to antibodies to EBV – VCA in patient with acute lymphoblastic leukemia and in the normal population, the results revealed large proportion of leukemic patients had raised antibodies to EBV viral capsid antigen. The percent significantly higher in ALL cases when compared to healthy control (67% vs 51%; $p < 0.05$) (Sazawal *et al*., 2000).

Study conduct to assess EBV miRNAs expression and their contribution to the CLL disease progression. To do this, we analyzed by different methods (small RNA sequencing analysis, qRT-PCR and miRNA In Situ Hybridization) the expression levels of EBV miRNAs in B-cells, plasma and paraffin-embedded bone marrow biopsy specimens from patients with CLL and determined their association with clinical outcome, the study shows found that EBV miRNA BHRF1-1 expression levels were significantly higher in the plasma of patients with CLL compared with healthy individuals (p b 0 · 0001) (Ferrajoli *et al*., 2015).

CHAPTER THREE

MATERIALS AND METHOD

CHAPTER THREE

MTERIALS AND METHODS

3.1. Study design

3.1.1. Type of study

This is a Cross- sectional study.

3.1.2. Study duration

This work was carried out in the period from Februarys to September 2016.

3.1.3. Study setting

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

3.1.4. Study population

Patients who had already diagnosed by hematologist as lymphoid leukemic.

3.2. Sample size

A total of 86 subjects of lymphoid leukemic were collected in this study.

3.3. Data collection

Information such as age, gender, type of lymphoid leukemia, stage of disease was collected from patients by questionnaire (appendix 1).

3.3.1. Data analysis

The data that collected from questionnaire and laboratory results were analyzed by statistical package for social sciences (SPSS) version 11.5 computerized program.

3.4. Ethical consideration

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

3.5. Laboratory methods

3.5.1. Specimens collection and preservation

Five ml of venous blood was collected using venous puncture technique. The collected blood was drawn into EDTA container, and then centrifuged at 3000 rpm for 5minutes. The plasma was separated into epindroff tubes and preserved at -21°C until used.

3.5.2. Analysis of specimens

The specimens were analyzed for semiquantitative detection of EBV IgG antibodies by commercially available enzyme- linked immmunosorbent assay 'EBV IgG ELISA' kit (Euroimmun Medizinische Labordiagnostika, D- 23560 Lubeck, seekamp 31 Germany).The assay was performed following the instruction

of the manufacture. According to the information included in the kit's insert, the immunoassay used has sensitivity 100% and specificity 100%.

3.5.3. Principle

The test kit contains microtiter strips each with 8 break-off reagent wells coated with EBV-VCA. 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 antigens. To detect the bound antibodies, a second incubation is carried out using an enzyme labeled anti-human IgG (enzyme conjugate) catalysing a colour reaction (appendix 2).

3.5.4. Procedure

1. All reagents were brought to room temperature approximately 30 minutes before used.

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

3. Samples were diluted by adding 10 µl to 1ml of samples diluents.

4. 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).

5. Washed the wells three times using 300µl diluted washing buffer.

6. 100µl of enzyme conjugate (peroxide-labeled anti-human IgG) were dispensed into all wells, incubated for 30 min at room temperature (18°C -25°C).

7. Washed the wells three times using 300 µl diluted washing buffer.

8. 100µl of chromogen/substrate (TMB-H_2O_2) solution was dispensed into all wells incubated for 15 min at room temperature (appendix 4).

9. 100µl of stop solution (0.5Msulphuric acid) was added to the wells containing chromogen /substrate solution(appendix 5).

10. The color intensity was read at wave length of 450nm within 30minutes of adding stop solution.

3.6. Quality control and Calculation of the result

3.6.1. Quality control

Reagents and calibrators are checked for storage, stability and preparation before starting work (appendix 3). .

1. Calibrator 1 absorbance must be 1.928 at 450 nm.
2. Calibrator 2 absorbance must be 0.282 at 450 nm.
3. Calibrator 3 absorbance must be 0.060 at 450 nm.
4. Positive control absorbance must be 3.2 at 450 nm.
5. Negative control absorbance must be 0.3 at 450 nm.

3.6.2. Calculation of results

Results were evaluated semiquantitatively 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:

$$Ratio = \frac{\text{Extinction of the contol or patint sample}}{Extinction of calibrator\ 2}$$

3.6.3. Interpretation of results

EURO IMMUN recommends interpreting results as follows:

Ratio <0.8: negative

Ratio ≥0.8to< 1.1: borderline

Ratio ≥1.1: positive

CHAPTER FOUR

RESULTS

CHAPTER FOUR

RESULTS

A total of eighty six(n=86) blood specimens were obtained from leukemic patients from Radiation and Isotope Center of Khartoum.

All specimens were examined for the presence of EBV IgG antibodies using ELISA kit. The results showed that from 86 blood specimens investigated, 81(94.2%) were positive for EBV, while the rest 5(5.8%) were negative(Table 1).56 (65.1%) in males and 25 (29.1%) in females (Table 2), with high prevalence among age group (1-20) 58 (93.5%) were positive, age group (21-40) 1(100%) were positive, and age group (41-60) 8(88.8%) were positive, and age group (61-80)14 (100%) were positive(Table 3).

High prevalence of EBV IgG 60 (93.7%) was detected among patients with acute lymphoid leukemia (ALL) and 21 (95.4%) in chronic lymphoid leukemia patients (CLL)(Table 4). According to disease stage the prevalence of EBV IgG among ALL patients in different stages was follows; 36 (97.2%) were positive in Pre B cell stage, 19 (86.3%) were positive in B cell stage and 6 (100%)were positive in T cell stage while the prevalence of EBV IgG among CLL patients was showed as follows; 5 (100%) were positive in stage I, 8 (88.8%) were positive in stage II and 8 (100%) were positive in stage III (Table 5).

Table 1. Result of EBV among leukemic patients

RESULTS	EBV	
	n.	%
Positive	81	94.2
Negative	5	5.8
Total	86	100

n: number

Table 2. Result of EBV according to gender

Target groups	n.	EBV	
		Positive	%
Males	59	56	65.1
Females	27	25	29.1
Total	86	81	94.2

P = 0.66

n: number

Table 3. Result of EBV according to age group

Age group	n.	EBV	
		Positive	%
(1 – 20)	62	58	93.5
(21 – 40)	1	1	100
(41 – 60)	9	8	88.8
(61 – 80)	14	14	100
Total	86	81	94.2

P = 0.69

n: number

Table 4.Result of EBV according to type of leukemia

Type of lymphoid leukemia	n.	EBV	
		Positive	%
ALL	64	60	93.7
CLL	22	21	95.4
Total	86	81	94.2

P = 0.76

n: number

Table 5. Result of EBV according to disease stage

Disease stage	n.	EBV	
		Positive	%
Pre B cell	37	36	97.2
B cell	22	19	86.3
T cell	6	6	100
Stage I	5	5	100
Stage II	9	8	88.8
Stage III	7	7	100
Total	86	81	94.2

P = 0.45

n: number

CHAPTER FIVE

DISCUSSION

CHAPTER FIVE

DISCUSSION

5.1 Discussion

Epstein- Barr virus 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 and Watkins, 2001).

In present study aimed to detect EBV among lymphoid leukemic patients in Khartoum State, out of 86 blood specimens investigated EBV was detected in leukemic patients irrespective to age group, gender and disease stage; however, there was a high frequency in patients with ALL 60 (93.7%) and 21 (95.4%) CLL. Hussain *et al*., (2012) conducted a study aimed to investigate the association of EBV in childhood leukemia in Sudan, the study shows that EBV was detected in 29 (36%) of the 80 patients of leukemia but in none of the healthy controls. Of the 29 EBV positive cases, 23 (79%) were acute lymphoblastic leukemia (ALL) (Hussain *et al*., 2012). These outcomes and the present findings show a marked association between EBV and ALL, Although the prevalence of EBV is higher in another study carried out by AbdELraheem in Khartoum State showed that the

prevalence of EBV infection among patients with ALL was 80% (21/26) and 72% (13/18) among patients with CLL (AbdELraheem, 2015).

Sazawal *et al* (2000) mentioned in their study that a large population of leukemia patients had raised antibodies to EBV viral capsid antigen. The percent was significantly higher in ALL cases when compared to healthy control (67% vs 51%; $P < 0.05$) (Sazawal *et al.*, 2000). This study collectively indicates the relation between EBV and ALL.

Finally, the frequency of EBV is higher in ALL than in CLL and according to the outcomes of this study, there was no significant variation in the frequency of EBV among the age groups, gender, stage of leukemia; this may be attributed to the high prevalence in developing countries mainly in adolescence and young adults.

5.2. Conclusion

Based on the results of this study, there is high prevalence rate of EBV infections among lymphoid leukemic patients in Khartoum.

Statistical analysis shows that there is significant association between EBV and lymphoid leukemia.

5.3. Recommendations

Screening of donor's blood and leukocyte filtration should be performed at the blood bank to reduce the risk of EBV transmission via blood.

Antiviral prophylaxis must be run to reduce the risk for EBV infection.

Health educational programs must be improved to facilitate in prevention and control of EBV infections.

Further studies and more advanced techniques are required to validate the results of the present study.

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APPENDICES

APPENDICES

Appendix (1): Questionnaire

Sudan University of Science and Technology

College of Graduate Studies

Seroprevalence of Epstein- Barr Virus among Lymphoid Leukemia Patients in Khartoum State

Name:…………………………………………………………………………………………

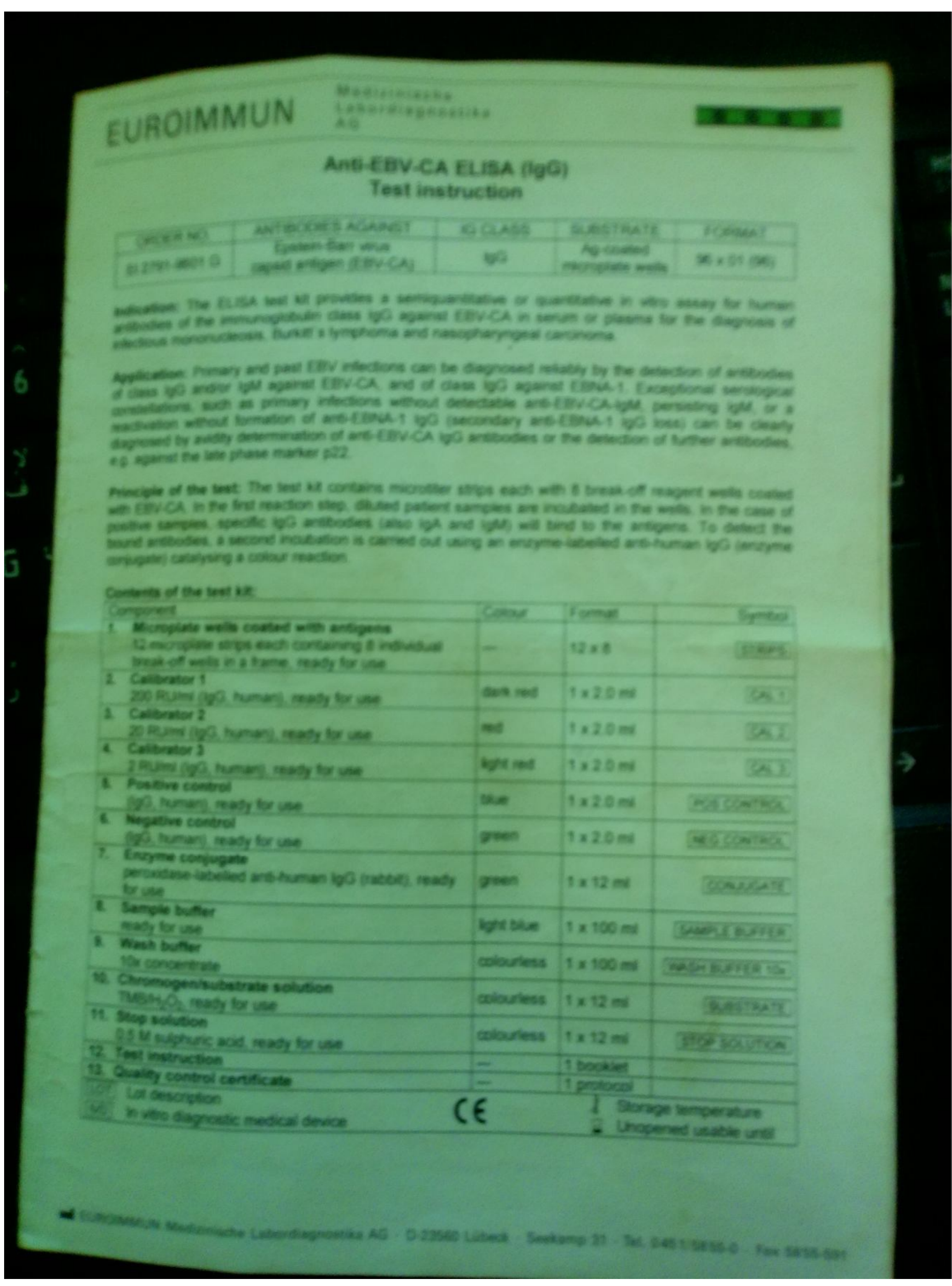
Age:……………………………………………………………………………………………..

Gender:………………………………………………………………………………………….

Type of leukemia:………………………………………………………………………………

Disease stage:……………………………………………………………………………………

Appendix (2): leaflet of anti-EBV –CA ELISA (IgG)



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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)

Indication: The ELISA test kit provides a semiquantitative or quantitative in vitro assay for human antibodies of the immunoglobulin class IgG against EBV-CA in serum or plasma for the diagnosis of infectious mononucleosis, Burkitt's lymphoma and nasopharyngeal carcinoma.

Application: Primary and past EBV infections can be diagnosed reliably by the detection of antibodies of class IgG and/or IgM against EBV-CA, and of class IgG against EBNA-1. Exceptional serological constellations, such as primary infections without detectable anti-EBV-CA-IgM, persisting IgM, or a reactivation without formation of anti-EBNA-1 IgG (secondary anti-EBNA-1 IgG loss) can be clearly diagnosed by avidity determination of anti-EBV-CA IgG antibodies or the detection of further antibodies, e.g. against the late phase marker p22.

Principle of the test: 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 description
IVD In vitro diagnostic medical device
CE
Storage temperature
Unopened usable until

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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 crystallisation 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 deionised 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.

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.

Warning: The calibrators and controls of human origin have tested negative for HBsAg, anti-HCV, anti-HIV-1 and anti-HIV-2. Nonetheless, all materials should be treated as being a potential infection hazard and should be handled with care. Some of the reagents contain the agent sodium azide in a non-declarable concentration. 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 of sample in 1.0 ml sample buffer and mix well by vortexing (sample pipettes are not suitable for mixing).

NOTE: The calibrators and controls are prediluted and ready for use, do not dilute them.

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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

<u>Sample incubation:</u> (1st step)	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).
<u>Washing:</u>	<u>Manual:</u> Empty the wells and subsequently wash 3 times using 300 µl of working strength wash buffer for each wash. <u>Automatic:</u> Wash the reagent wells 3 times with 450 µl working strength wash buffer (program setting: e.g. TECAN Columbus Washer "Overflow Mode"). Leave the wash buffer in each well for 30 to 60 seconds per washing cycle, then empty the wells. After washing (manual <u>and</u> 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. <u>Note:</u> 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 residence 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.
<u>Conjugate incubation:</u> (2nd step)	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).
<u>Washing:</u>	Empty the wells. Wash as described above.
<u>Substrate incubation:</u> (3rd step)	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).
<u>Stopping:</u>	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.
<u>Measurement:</u>	**Photometric measurement** of the colour intensity should be made at a **wavelength of 450 nm** and a reference wavelength between 620 nm and 650 nm **within 30 minutes of adding the stop solution**. Prior to measuring, slightly shake the microplate 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 an 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 enquiry.
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	[illegible]	[illegible]	[illegible]	[illegible]			[illegible]	[illegible]	[illegible]	[illegible]		
B	pos.	[illegible]	[illegible]	[illegible]			[illegible]	[illegible]	[illegible]	[illegible]		
C	neg.	[illegible]	[illegible]	[illegible]			[illegible]	[illegible]	[illegible]	[illegible]		
D	[illegible]	[illegible]	[illegible]				pos.	[illegible]	[illegible]	[illegible]		
E	[illegible]	[illegible]	[illegible]				neg.	[illegible]	[illegible]	[illegible]		
F	[illegible]	[illegible]	[illegible]				[illegible]	[illegible]	[illegible]			
G	[illegible]	[illegible]	[illegible]				[illegible]	[illegible]	[illegible]			
H	[illegible]	[illegible]	[illegible]				[illegible]	[illegible]	[illegible]			

The pipetting protocol for microtiter strips 1 to 4 is an example for the **semiquantitative analysis** of 24 patient samples (P 1 to P 24).
The pipetting protocol for microtiter strips 7 to 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 wells can be broken off individually from the strips. Therefore, the number of tests performed can be matched to the number of samples, minimising 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

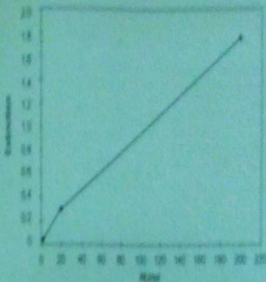
Semiquantitative: Results can be evaluated semiquantitatively by calculating a ratio of the extinction value of the control or patient sample over the extinction value of the calibrator 2. Calculate the ratio according to the following formula:

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

EUROIMMUN recommends interpreting results as follows:

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

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 for a patient sample lies above the value of calibrator 1 (200 RU/ml), the result should be reported as ">200 RU/ml". It is recommended that the sample be retested at a dilution of e. g. 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, EUROIMMUN recommends retesting the samples.

A negative serological result does not exclude an infection. Particularly in the early phase of an infection, antibodies may not yet be present or are only present in such small quantities that they are not detectable. In case of a borderline result, a secure evaluation is not possible. If there is a clinical suspicion and a negative test result, we recommend clarification by means of other diagnostic methods and/or the serological investigation of a follow-up sample. A positive result indicates that there has been contact with the pathogen. In the determination of pathogen-specific IgM antibodies, polyclonal stimulation of the immune system or antibody persistence may affect the diagnostic relevance of positive findings. Significant titer increases (exceeding factor 2) and/or seroconversion in a follow-up sample taken after 7 to 10 days can indicate an acute infection. To investigate titer changes, sample and follow-up sample should be incubated in adjacent wells of the ELISA microplate within the same test run. For diagnosis, the clinical picture of the patient always needs to be taken into account along with the serological findings.

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Interpretation of serological constellations in EBV diagnostics

		EBV-CA IgG	gp125 and/or p19 IgG	EBV-CA IgM	gp125 and/or p19 IgM	EBV-EA IgG	EBNA	IgG-avidity
No infection with EBV		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Infectious mononucleosis	Early phase	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	Late phase	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	low
	Convalescence	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	high
	Infection a long time ago	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	high
	Reactivation	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	high
	Chronic	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	high
Special serological constellations	Early phase without detectable anti-CA IgM or retarded formation of anti-IgM [?]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	low
	Past infection with persisting anti-VCA IgM	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	high
	Recent infection with IgM reactivation	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	high
	Early phase without detectable anti-EA IgG	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	low
	Past infection with anti-EBNA-1 loss	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	high
	Past infection with retarded or absent anti-EBNA-1 conversion	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	high
Burkitt's lymphoma		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	high
Nasopharyngeal carcinoma (EBV-CA IgA [illegible] EBV-EA IgA [illegible])		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	high
Reactivated infection		Determination of the viral load is indicated for characterisation of reactivated EBV infections in immunosuppressed patients or persons with a weakened immune system. In these cases the serological results are of limited relevance compared to the determination of the viral load.						

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 binding activity of the antibodies and the activity of the enzyme used are temperature-dependent. It is therefore recommended using a thermostat in all three incubation steps. The higher the room temperature (+18°C to +25°C) during the incubation steps, the greater will be the extinction values. Corresponding variations apply also to the incubation times. However, the calibrators 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 lower detection limit of the Anti-EBV-CA ELISA (IgG) is 5.9 RU/ml.

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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) positive
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 on the result up to a concentration of 10 mg/ml for haemoglobin, 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 (CV) using 3 samples. 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		
Sample	Mean value (RU/ml)	CV (%)
1	47	7.4
2	90	5.8
3	93	4.2

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

Sensitivity and specificity: 175 clinically pre-characterised patient samples (INSTAND and Labquality) were investigated with the EUROIMMUN Anti-EBV-CA ELISA (IgG). The sensitivity amounted to 100%, with a specificity of 100%. Borderline results were not included in the calculation.

n = 175		INSTAND/Labquality		
		positiv	borderline	negativ
EUROIMMUN Anti-EBV-CA ELISA (IgG)	positive	145	0	0
	borderline	3	1	0
	negative	0	0	26

Reference range: The levels of the anti-EBV-CA antibodies (IgG) were analysed 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) is one of the most widely distributed human-pathogenic herpes viruses. The virus is transmitted by smear infection, but also by blood transfusions or organ transplants. EBV is the causative agent of infectious mononucleosis (Pfeiffer's disease), a febrile disease usually accompanied by pharyngitis and lymphadenopathy, frequently by hepatosplenomegaly and more rarely by an exanthema. When first infection occurs in childhood the disease often proceeds without symptoms. In industrial countries mainly adolescents or young adults become infected, often leading to manifest disease. EBV infections are also found in connection with the pathogenesis of malignant lymphoma (endemic form of Burkitt's lymphoma in Africa) and nasopharyngeal carcinoma (NPC, especially widespread in South-East Asia). NPC is the third most frequent malignant tumour in southern China. Since 2011 the evidence has been increasing that EBV infection is associated with a high risk of multiple sclerosis (MS) or an aggravation of MS.

The main goal of EBV diagnostics in people with a healthy immune system is to differentiate between an acute and a past infection. Various serological methods are used for this. The immune system of healthy persons can quickly suppress a reactivation of the virus. However, in immunosuppressed patients (e. g. those on immunosuppressive therapy after organ transplantation or with an HIV infection) EBV can spread uncontrollably and cause severe lymphoproliferative diseases. In such cases it is diagnostically very important to also determine the viral load, for which PCR (polymerase chain reaction) is normally used.

Infectious mononucleosis must be differentiated from cytomegalic inclusion body disease and toxoplasmosis and, in the case of atypical progress, also from HIV infection or other infections.

In pregnancy, EBV can cause infection of the placenta, leading to damage to the foetal heart, eyes and liver. In children, accompanying infections of the kidney have been observed with symptoms from microscopic haematuria to acute kidney failure.

The immune response to an EBV infection is characterised by the formation of antibodies against EBV capsid, EBV nuclear and EBV early antigens successively.

In the early phase of the disease IgM and IgG antibodies against the viral capsid antigen (CA) can be detected. A positive Anti-EBV-CA (IgM) result is the classical marker of acute infection. IgG antibodies against early antigen are produced later in the acute phase and decrease after three to six months to a non-detectable level. Anti-CA IgG antibody levels persist lifelong. Around six to eight weeks after infection, antibodies against EBNA are produced. The presence of anti-EBNA antibodies indicates a past infection.

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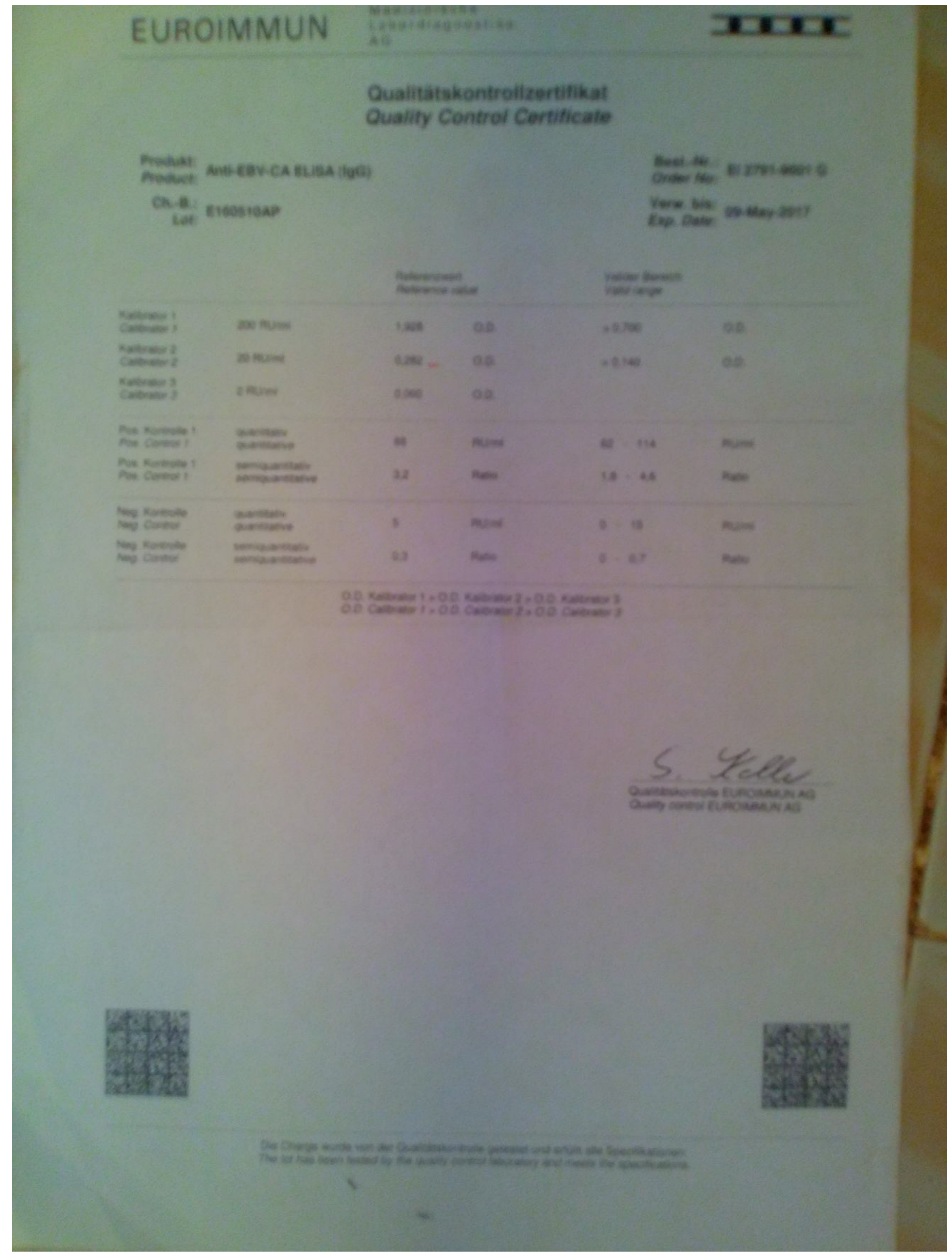
Appendix (3): Quality control leaflet of anti-EBV –CA ELISA (IgG)



EUROIMMUN

Qualitätskontrollzertifikat
Quality Control Certificate

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

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

Ch.-B.: / *Lot:* E160510AP

Verw. bis: / *Exp. Date:* 09-May-2017

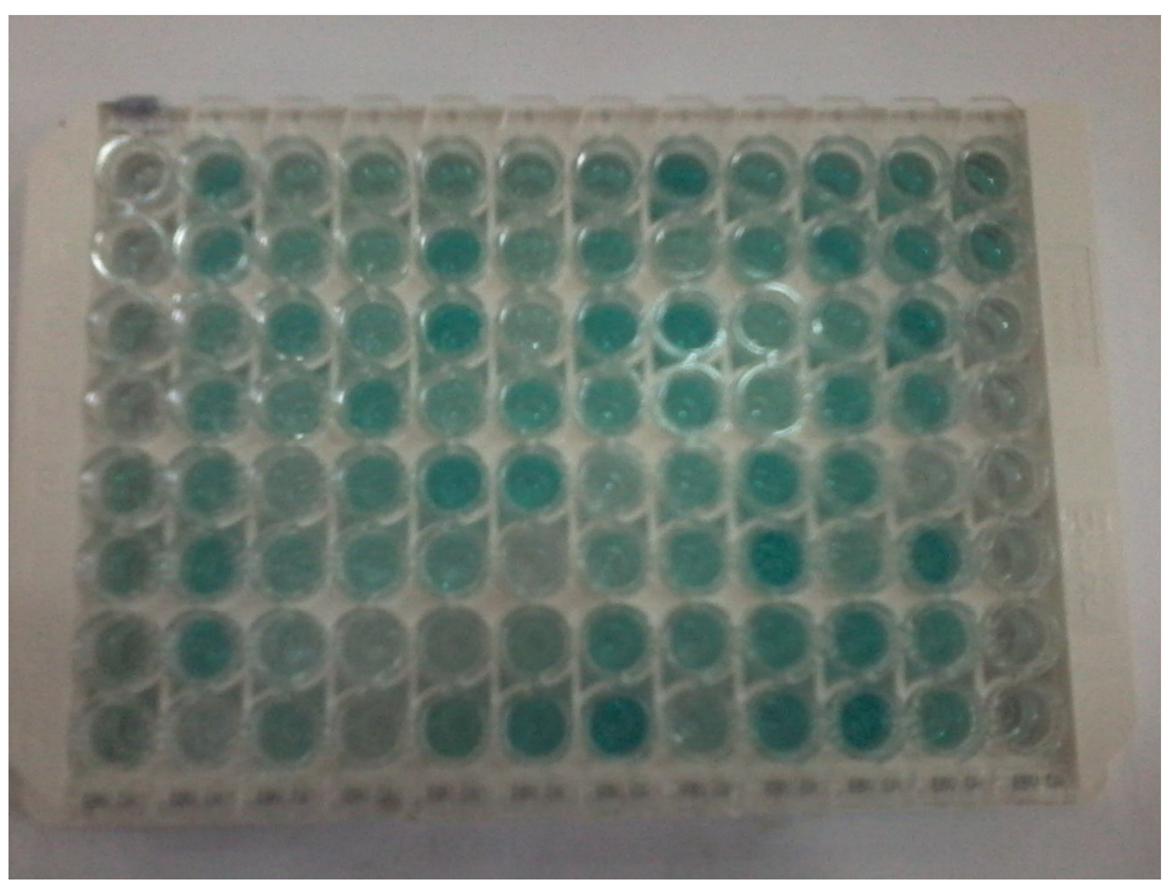
		Referenzwert / *Reference value*		Valider Bereich / *Valid range*	
Kalibrator 1 / *Calibrator 1*	200 RU/ml	1,928	O.D.	> 0,700	O.D.
Kalibrator 2 / *Calibrator 2*	20 RU/ml	0,282	O.D.	> 0,140	O.D.
Kalibrator 3 / *Calibrator 3*	2 RU/ml	0,060	O.D.		
Pos. Kontrolle 1 / *Pos. Control 1*	quantitativ / *quantitative*	88	RU/ml	62 - 114	RU/ml
Pos. Kontrolle 1 / *Pos. Control 1*	semiquantitativ / *semiquantitative*	3,2	Ratio	1,8 - 4,6	Ratio
Neg. Kontrolle / *Neg. Control*	quantitativ / *quantitative*	5	RU/ml	0 - 15	RU/ml
Neg. Kontrolle / *Neg. Control*	semiquantitativ / *semiquantitative*	0,3	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

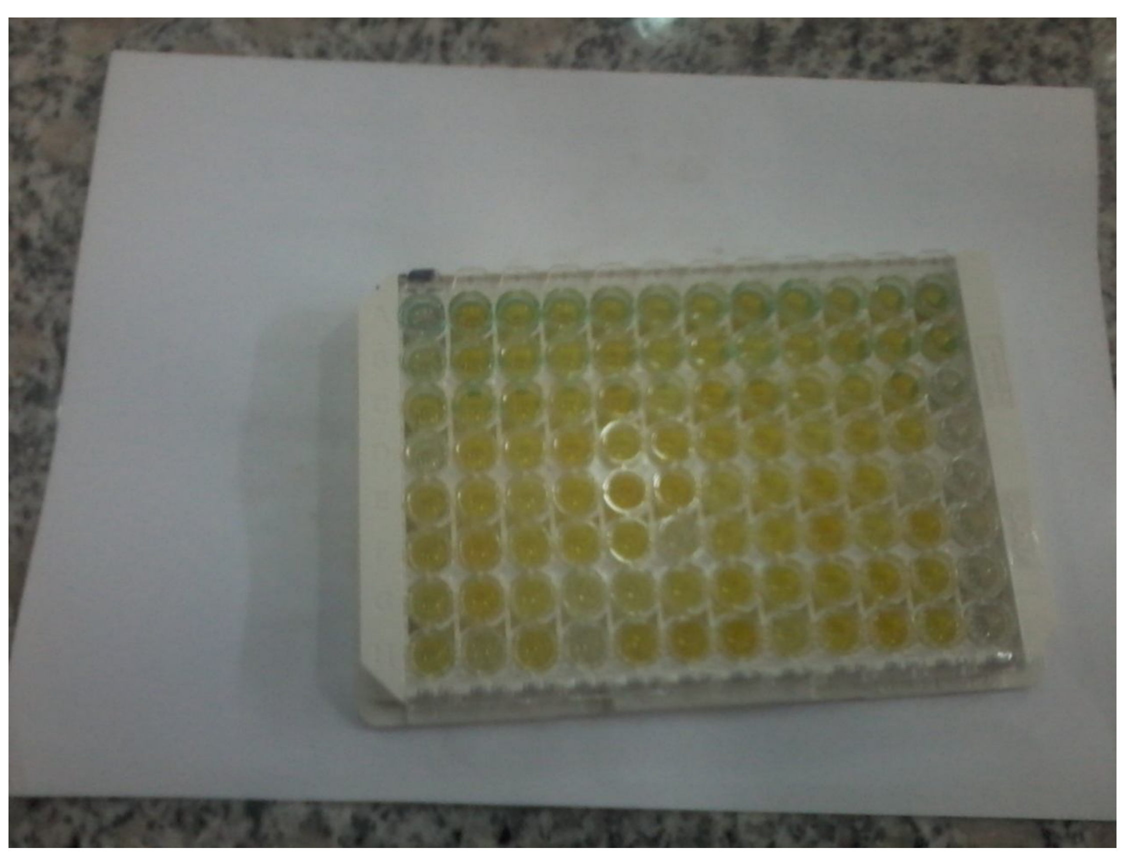
Qualitätskontrolle EUROIMMUN AG
Quality control EUROIMMUN AG

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.

Appendix (4): microplate well of ELISA before pipetting the stop solution (0.5 M sulphuric acid



Appendix (5): microplate well of ELISA after pipetting the stop solution (0.5 M sulphuric acid



Difference data

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.017	2.718	1.174	1.039	1.396	1.157	1.321	3.169	1.603	2.093	1.993	1.233
B	0.284	2.901	1.564	1.217	3.18	1.202	2.268	0.768	2.012	3.093	2.483	1.939
C	1.12	1.776	2.846	1.404	3.309	0.578	3.191	3.334	1.085	1.19	3.415	0.04
D	0.148	2.565	1.238	2.87	1.169	2.448	2.055	2.332	1.006	2.438	2.417	0.043
E	1.796	2.227	0.622	1.911	3.346	3.07	0.648	1.511	2.73	2.172	0.056	0.031
F	1.867	3.195	1.44	1.533	1.443	0.078	1.093	1.568	3.391	0.781	3.413	0.092
G	1.012	3.065	0.823	0.263	0.414	0.854	2.085	1.395	1.893	2.569	2.006	0.039
H	1.224	0.263	1.602	0.098	1.953	2.591	3.402	0.475	2.417	3.281	2.197	0.034

N-ve = $\frac{0.148}{0.284}$ = 0.521

P+ve = $\frac{1.12}{0.284}$ = 3.94

64 22
ALL + CLL