Sudan University of Science & Technology

College of Graduate Studies

Serodetection of *Epstein-Barr Virus* (EBV) among Systemic Lupus Erythematosus Patients in Khartoum State

الكشف المصلي لفيروس ابشتاين بار وسط مرضى الذئبة الحمراء في ولاية الخرطوم

A dissertation submitted in partial fulfillment for the requirements of M.Sc. degree in Medical Laboratory Science (Microbiology)

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الآيــــة

قال تعالى:

﴿ قُل بِفَضلِ اللَّهِ وَبِرَحمَتِهِ فَبِذلِكَ فَليَفرَحوا هُوَ خَيرٌ مِمّا يَجمَعونَ ﴾

صدق الله العظيم

سورة يونس، الآية (٥٨)

DEDICATION

To my father and mother

Who gave me strength and encouragement, love and kindness

To my lovely sisters and brothers

Who gave me happiness and joy

To my best friends

Who supported me

To myself

That endured the difficulties and passed many obstacles to accomplish this research

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Thanks and praise to **ALMIGHTY ALLAH** who gave me the strength, health and patience to achieve this work.

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ABSTRACT

Epstein Barr virus (EBV) is associated with genesis of many human autoimmune diseases, especially Systemic Lupus Erythematosus (SLE). Many studies have revealed a connection between SLE and EBV infection, which show increased levels of EBV-directed antibodies.

This case-control study was aimed to detect EBV serologically among Sudanese SLE patients and healthy subjects. It was conducted at Military Hospital, during the period October 2019 to March 2021.

A total of 92 paticipants (n=92) were included in this study with age ranged from 20-70 years with an average mean of 36.3±11.7 S.D, in which there were 46 patients with SLE and 46 apparently healthy persons matched in age and gender as controls.

From the study participants, blood samples were collected and analyzed by Enzyme-Linked Immunosorbent Assay (ELISA) for the detection of IgM antibodies to EBV-Viral Capsid Antigens.

Out of the 46 SLE patients 15/46 (32.6%) were positive for IgM anti-EBV-VCA antibodies, while 7/46 (15.2%) were positive among non-SLE individuals.

Regarding age group, EBV was most frequent in age group 20 to 35 years among SLE patients and non-SLE individuals (7/15 (46.7%) and 6/7 (85.7%) respectively). There was no significant association between age and EBV infection in SLE patients (P=0.857) and non-SLE individuals (P=0.089).

According to gender, EBV infection was found among SLE patients as follow: 10/15 (66.7%) were females and 5/15 (33.3%) were males positive to anti−EBV VCA with significant association (P=0.017) between gender and EBV infection. While non-SLE individuals, there were 6/7 (85.7%) males and 1/7 (14.3%) females were positive for IgM anti-EBV-VCA antibodies with significant association (P=0.040) between gender and EBV infection.

From the above findings, the study concluded that the recent EBV infection was common among SLE patients and had high frequency among females. Hence, further researches must be carried out to determine the association between EBV and SLE as causal relationship.

ملخص الأطروحة

يرتبط فيروس إبشتاين بار مع نشوء العديد من أمراض المناعة الذاتية البشرية، وخاصة الذئبة الحمراء. كشفت العديد من الدراسات عن وجود صلة بين الإصابة بمرض الذئبة الحمراء و فيروس إبشتاين بار، والتي تُظهر مستويات متزايدة من الأجسام المضادة الموجهة إلى الفيروس.

هدفت هذه الدراسة (الحالة–الضابطة) إلى الكشف عن فيروس إبشتاين بار مصليا بين المرضى السودانيين المصابين بمرض الذئبة الحمراء مقارنة بمجموعة من الأصحاء. وأجريت في المستشفى العسكري، خلال الفترة من أكتوبر ٢٠١٩ إلى مارس ٢٠٢١.

أجريت هذه الدراسة على ٩٢ شخص (ن=٩٢) تتراوح أعمارهم بين ٢٠–٧٠ سنة بمتوسط قدره ٣,٣٦±٧,١١ إنحراف معياري، حيث أن ٤٦ منهم مصاب بالذئبة الحمراء و٤٦ أشخاص أصحاء مطابقين في العمر والجنس.

تم جمع عينات الدم من المشاركين في الدراسة وتحليلها بواسطة الفحص المناعي المرتبط بالإنزيم للكشف عن الأجسام المضادة من النمط IgM إلى المستضدات القفيصة الفيروسية الخاصة بفيروس إبشتاين بار.

من بين ٤٦ من مرضى الذئبة الحمراء الذين شاركوا في الدراسة ، ٤٦/١٥ (٦,٣٢٪) أعطوا نتائج إيجابية للأجسام المضادة النمط IgM، و٤٦/٧ (١٥,٢٪) بين مجموعة الأشخاص الأصحاء.

فيما يتعلق بالعمر، كان فيروس إبشتاين بار أكثر انتشارا في الفئة العمرية ٢٠ إلى ٣٥ سنة وسط مرضى الذئبة الحمراء والأصحاء (١٥/٧ (٧,٤٦٪) و ٧/٦ (٨٥,٧٪)على التوالي). وليس هنالك علاقة ذات قيمة معنوية بين العمر و الإصابة بفيروس إبشتاين بار وسط مرضى الذئبة الحمراء (القيمة الاحتمالية=٠,٨٥٧) والغير مصابين بالذئبة الحمراء (القيمة الاحتمالية=٠,٠٨٩).

فيما يخص الجنس: وجدت أن الإصابة بفيروس إبشتاين بار وسط مرضى الذئبة الحمراء كالاتي: ١٥/١٠ (٧,٦٦٪) إناث و١٥/٥ (٣,٣٣٪) ذكور أعطوا نتيجة إيجابية لمستضدات القفيصة الفيروسية الخاصة بفيروس إبشتاين بار مع وجود علاقة ذات دلالة إحصائية بين الجنس والإصابة بفيروس ابشتاين بار (القيمة الاحتمالية=٠,٠١٧). بينما في الأصحاء كان هنالك ٧/٦ (٧,٨٥٪) ذكور و ٧/١ (٣,١٤٪) إناث أعطوا نتيجة لمستضدات القفيصة الفيروسية الخاصة بفيروس إبشتاين بار مع وجود علاقة ذات دلالة إحصائية بين الجنس والإصابة بفيروس ابشتاين بار (القيمة الاحتمالية=٠,٠٤٠).

من النتائج المذكورة أعلاه خلصت الدراسة إلى أن عدوى فيروس إبشتاين بار الحديثة أكثر شيوعا وسط مرضى الذئبة الحمراء في السودان وأكثر انتشارا وسط الإناث. وبالتالي، يجب إجراء المزيد من البحوث لتحديد العلاقة بين فيروس إبشتاين بار و مرض الذئبة الحمراء كعلاقة سببية.

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LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
ANAs	Antinuclear Antibodies
BL	Burkitt ’s lymphoma
DNA	Deoxyribonucleic Acid
EA	Early Antigens
EBNA	Epstein - Barr virus Nuclear Antigens
EBV	Epstein - Barr virus
EIA	Enzyme Immune Assay
ELISA	Enzyme-Linked Immunosorbent Assay
EMBASE	Excerpta Medica dataBASE
HA	Heterophile Antibodies
HD	Hodgkin’s Disease
IG	Immunoglobulins
IM	Infectious Mononucleosis
INF	Interferon
LMP	Latent Membrane Proteins
LP	Leader Protein
LPD	Lymphoproliferative Disorders
LYDMA	Lymphocyte Detected Membrane Antigens
MA	Membrane Antigen
NPC	Nasopharyngeal Carcinoma
OD	Optical Density
PCNSL	Primary Central Nervous System Lymphoma
PCR	Polymerase Chain Reaction
PTLD	Post Transplant Lymphoproliferative Disease
SLE	Systemic Lupus Erythematosus
TNF	Tumor Necrosis Factor
VCA	Viral Capsid Antigen
XLP	X-Linked Lymphoproliferative Disease

CHAPTER I

INTRODUCTION

CHAPTER I

1. INTRODUCTION

1.1. Introduction

Epstein-Barr virus (EBV) is a DNA virus that belongs to the human herpes virus family and infects 75% of the population before the age of 5 years and most humans are infected in childhood by saliva transfer and become lifelong carriers of EBV (Ahmed *et al*., 2011).

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

Systemic Lupus Erythematosus is a disease with periods of waning disease activity and intermittent flares. Characterized by widespread immune dysregulation with hyperproduction of numerous autoantibodies and immune complexes, resulting in chronic systemic inflammation and potential damage to multiple organs. The etiology of SLE is not fully established, it is may occurs when an environmental trigger induces an immunological dysfunction in a genetically predisposed individual, leading to the loss of tolerance towards native proteins. Among the environmental triggers, viruses including EBV, cytomegalovirus, parvovirus B19 and human endogenous retroviruses were postulated. According to different studies, the features of disease fits well in theory to a latent EBV infection which occasionally switches to lytic cycle, and EBV infection has for long been suspected to be involved (Draborg *et al*., 2012; Elsaied *et al.*, 2016). In several studies, SLE patients have been shown to have at least a 10 folds increased frequency of EBV-infected peripheral B cells, in addition to an abnormally high viral load in the peripheral blood mononuclear cells (PBMCs) compared to healthy individuals. This increase is associated with increased disease activity in SLE patients (Draborg *et al*., 2012).

Earlier studies have reported a functionally impaired EBV specific CD8+T-cell response, characterized by the decreased production of cytokines (interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-2 and macrophage inflammatory protein-1β) and decreased cytotoxicity in SLE patients (Berner *et al*., 2005).

1.2. Rationale

Epstein-Barr virus (EBV) is an environmental factor associated with SLE and it maintains latency in B cells with frequent reactivation but its relationship with SLE is questionable. Multiple investigators have suggested a strong association between previous EBV infection and SLE among children and adults in different populations (Elsaied *et al.*, 2016).

The association between EBV infection and SLE disease has attracted considerable attention in recent years, but to my knowledge there were few studies done in Sudan concerning this association, so this study was designed to spotlight on the frequency of EBV virus among SLE patients in Khartoum State, Sudan. So, data from this study could be helpful as baseline for further studies in such area.

1.3. Objectives

1.3.1. General objective

To detect EBV serologically among SLE patients and healthy control subjects in Khartoum State.

1.3.2. Specific objectives

1. To detect anti-EBV capsid antigen (IgM antibodies) by enzyme-linked immunosorbent assay (ELISA) among SLE patients and individuals without SLE (controls).
2. To determine the frequency of EBV infection among SLE patients and non-SLE subjects.
3. To determine the possible association between gender and age with EBV infection among SLE patients and individuals without SLE.

CHAPTER II

LITERATURE REVIEW

CHAPTER II

2. LITERATURE REVIEW

2.1. Epstein Barr Virus

2.1.1. Historical Background

Epstein-Barr virus (EBV) was discovered as a result of pioneering work in the 1950s by Denis Burkitt who identified a previously unrecognized form of cancer which affected the jaws of young African children, and he made the crucial insight that the distribution of this common tumor (now known as Burkitt's lymphoma) appeared to be influenced by climatic factors-notably temperature and elevation. His work was followed by Tony Epstein, an English pathologist. Epstein with his graduate student Yvonne Barr identified by electron microscopy a novel herpes virus in lymphoma cell cultures (Swanson, 2007).

In 1968 EBV was shown to be the etiologic agent of heterophil positive infectious mononucleosis. EBV DNA was detected in tissue from patients with nasopharyngeal carcinoma in 1970. In the 1980 EBV was found to be associated with non-Hodgkin's lymphoma and oral hairy leukoplakia in patients with acquired immunodeficiency syndrome (AIDS). Since then EBV DNA has been found in tissues from other cancers including T-cell lymphomas and Hodgkin's disease (Cohen, 2000).

2.1.2. Classification

EBV is a member of the Herpesviridae family, subfamily Gamma herpesvirinae, genus Herpesvirus, displays two strain have been defined type-1 and type-2 (alternatively named A and B). EBV-1 is more prevalent worldwide than EBV-2, which is found more frequently in Africa (Jenson, 2011).

2.1.3. Morphology

Mature virions are approximately 120 to 180 nm in diameter surrounded by a protein capsid. A protein tegument lies between the capsid and the envelope, which is embedded with glycoproteins that are important for cell tropism, host range, and receptor recognition (Odumade *et al.*, 2011).

2.1.4. EBV genome

The EBV genome is a linear double-stranded DNA molecule, composed of somewhere around 85 genes (Battista, 2019).

2.1.5. EBV antigens

Lytic phase antigens which include early antigens (EA) that initiate replication, characterized as diffuse (D) and restricted (R). In addition to the late antigens which consist of viral capsid antigen (VCA) and membrane antigen (MA), which are structural components of the viral particle (Johannsen *et al*., 2004).

Viral capsid antigen (VCA) is a protein composed of a 110 kDa glycoprotein (gp110) which involved in virus maturation and improves the efficiency of the virus to infect B cells and epithelial cells (Draborg *et al*., 2012).

Latent phase antigens are the EB nuclear antigen (EBNA) and the latent membrane protein (LMP), EBNAs subsequently identified as six separate proteins (EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C and leader protein (LP) (Bazot *et al*., 2018).

EBNA1 protein has to be expressed in all EBV-infected proliferating cells because its main function is to ensure the persistence of the viral genome in the cells as they multiply (Farrell, 2019). EBNA2 is a transcription factor that directly activates viral LMP genes and approximately 300 cell genes including the B cell activation antigens (Zhao *et al*., 2006).

The EBNA3 family of proteins act as regulators of gene expression, repressing some genes and activating others, comprises EBNA3A, EBNA3B, and EBNA3C. Functionally, EBNA3A and EBNA3C tend to cooperate and contribute to the EBV transformation of B cells, whereas the functions of EBNA3B tend to be different (Farrell, 2019).

Latent Membrane Proteins (LMPs) recognized as the primary transforming gene product of the virus. It is expressed in most EBV-associated lymphoproliferative diseases and malignancies, and it critically contributes to pathogenesis and disease phenotypes (Kieser and Sterz, 2015).

2.1.6. Epidemiology

Over 90% of people are infected by EBV and most of them are asymptomatic, especially infants and children less than 6 years old, 25-30% of primary infection in adolescents and adults (Gao *et al*., 2011).

2.1.7. EBV transmission

EBV is transmitted primarily by oral contact with saliva, most commonly by exchange of saliva among young children directly or through the handling of toys or by kissing among adolescents, accounting for infectious mononucleosis commonly being called “kissing disease” (Jenson, 2011).

Also, it can spread through the blood, by means of blood transfusion and organ transplantations. Infected epithelial cells can also be found in the uterine cervix or in the semen, suggesting the possibility of EBV spread through sexual contact (Smatti *et al*., 2018).

2.1.8. EBV life cycle

Either to be a lytic cycle or a latent cycle (Swanson, 2007).

2.1.8.1. EBV lytic cycle

EBV replication occurs most efficiently in epithelial cells and also occurs spontaneously in a small fraction of the latently infected B cell population, as the result of viral reactivation. The signals responsible for this are unknown *in-vivo*, although *in-vitro* EBV replication can be triggered in a fraction of latently infected B cell (Swanson, 2007).

2.1.8.2. EBV latent cycle

EBV has ability to lie dormant within a cell, in which after initial infection virus production ceases. Latent Gene Expression is by six EBV nuclear antigens (EBNAs) and three latent membrane proteins (LMPs) which are expressed in latently infected cell lines. Five of the nine proteins have been shown to be essential for lymphocyte immortalization *in-vitro*. LMP-1 is the viral oncoprotein responsible for the majority of growth altering effects of EBV on B lymphocytes (Swanson, 2007).

2.1.9. EBV pathogenesis

Following uptake of virus by oral cavity, the EBV firstly infect oral epithelial cells then replicate causing cell lysis and produce virions which spread to adjacent structures such as salivary glands. This local replication may be the basis of the symptoms of pharyngitis. Viremia ensues, with infection of the primary cellular target of EBV, the B lymphocytes in the peripheral blood and the entire lymphoreticular system including the liver and spleen (Jenson, 2011).

2.1.10. Immunity to EBV

2.1.10.1. Humoral Immunity

A humoral immune response is also initiated during EBV infection, and EBV-infected individuals have distinct serologic profiles during the latent and acute phases. In early stages of the primary infection, IgM antibodies toward EBV-VCA and IgG antibodies to EBV-EA/D are generated, whereas EBNA-1 antibodies develop later. Serum IgA antibodies toward the EBV-EA/D have been shown to be produced during active disease and are suggested to be stimulated by EBV replication in mucosal sites (Draborg *et al.*, 2012).

VCA-IgM is detected in the active phase of infection and then declines in convalescence. VCA-IgG increases at the same time of VCA-IgM, but it remains positive for life indicating past infection. EBNA antibodies are detectable late in the phase of infection and also remain positive. Early antigens (EA) antibodies to the class R or D increase in the acute phase of infection and decline after convalescence (Smatti *et al.*, 2018).

2.1.10.2. Cellular Immunity

One of the most distinctive features of EBV infection is the presence of a leukocytosis of 10 to 20×10^3/"L (10 to 20×10^9/L), with atypical lymphocytes in the peripheral blood at the time of onset of clinical symptoms. Atypical lymphocytes often account for 20% to 40% of the total white blood cells (Jenson, 2011).

The expansion of EBV-infected B cells during lytic cycle is especially controlled by activated $CD8^+$ cytotoxic T cells, which kill infected B cells and also induce the latent state in remaining EBV-infected B cells. Cell-mediated immunity is also crucial in preventing the latent infection from entering lytic replication (Draborg *et al*., 2012).

2.1.11. EBV associated diseases

EBV is associated with several disease states, in some of which it is the direct etiological agent, whilst in others it acts as an essential co-factor in a complex series of events which lead to the following diseases (Lossisus *et al*., 2012).

2.1.11.1. Autoimmune diseases

Some data suggest that the EBV is associated with several autoimmune diseases, such as SLE, rheumatoid arthritis and multiple sclerosis. It is not clear whether EBV plays a role in the pathogenesis and if so, by which mechanisms the virus may contribute (Lossisus *et al.*, 2012).

2.1.11.2. Infectious mononucleosis (IM)

Is a clinical entity characterized by sore throat, cervical lymph node enlargement, fatigue and fever most often seen in adolescents and young adults and lasting several weeks resulting from primary infection with EBV. It is exceptionally low in Southeast Asia and equatorial Africa (Dunmire *et al*., 2015).

2.1.11.3. Burkitt's lymphoma (BL)

It is a cancer of the lymphatic system (in particular, B lymphocytes), is a highly aggressive lymphoma that is usually found in extra nodal sites or presenting as acute leukemia (Paraskevas and Dimitroulopoulos, 2005; Liu *et al*., 2007; Okano and Gross, 2012).

2.1.11.4. Nasopharyngeal carcinoma (NPC)

Is a malignant tumor thought to be derived from EBV infected epithelial cell (Dunmire *et al*., 2015).

2.1.11.5. Hodgkin's disease (HD)

It is an uncommon cancer that develops in the lymphatic system. The infected cells are starting to multiply in abnormal ways and begin to collect in certain parts of the lymphatic system, such as the lymph nodes (glands) (Paraskevas and Dimitroulopoulos, 2005; Okano and Gross, 2012).

2.1.11.6. X-linked lymphoproliferative disease (XLP) (Duncan's syndrome)

Stems from an inherited, maternally derived recessive genetic defect clinically manifest as three phenotypic expressions in boys 2-5 years of age, the most common of which is a fatal infectious mononucleosis. Burkitt-like lymphomas and persistent gamma globulinemia are the other phenotypes (Swanson, 2007).

2.1.11.7. Post-transplant lymphoproliferative disease (PTLD)

Is a well-recognized risk of immunosuppressive therapy associated with solid organ transplantation or T-cell depleted allogeneic bone marrow transplantation. PTLD behaves aggressively (Swanson, 2007).

2.1.11.8. Primary central nervous system lymphomas of AIDS (PCNSL)

These tumors are almost 100% EBV associated. The disease is usually fatal within two months of diagnosis (Swanson, 2007).

2.1.11.9. Oral hairy leukoplakia

Is a benign, asymptomatic, white, hyperkeratotic lesion affecting primarily the lateral border of the tongue, unilaterally or bilaterally, caused by productive replication of EBV in the oral mucosal epithelium, particularly of the lateral borders of the tongue. Its surface may be flat, vertically corrugated, or frankly hairy, and it affects severely immunocompromised subjects, most notably those infected with HIV (Khammissa *et al*., 2016).

2.1.11.10. Kawasaki's disease (mucocutaneous lymph node syndrome of children)

It is associated with potential lethal coronary artery aneurysms. A relatively high incidence of EBV DNA sequences in peripheral blood mononuclear cells indicates an association to EBV (Swanson, 2007).

2.1.12. EBV infection markers

The heterophil antibodies (HA); they are IgM class antibodies directed against mammalian erythrocytes and most commonly used to diagnose infectious mononucleosis (Dunmire *et al.*, 2015).

Early antigen (EA) D/R; antibody to EA may be detected in the majority of patients with acute EBV infection, then falls to a low or undetectable level. IgG antibodies against EA are detected transiently in up to 3 months or more (Smatti *et al.*, 2018).

Viral capsid antigen (VCA); detecting presence of IgM antibodies to VCA is virtually diagnostic of acute EBV infection, which usually disappears after convalescence and generally it does not occur another time in life. Whereas IgG anti-VCA is found in acute, convalescence, or past infections, as it starts to appear at the same time as VCA-IgM. Detection of VCA IgG on a single sampling indicates only that infection with EBV has occurred sometime in the past (Smatti *et al.*, 2018).

Epstein-Barr virus Nuclear antigen1 (EBNA1); its antibodies appear later in the course of EBV infection. EBNA-1 IgG antibodies appear late, 3 to 6 months after the time of disease, and then they decline but continue to be present in a detectable level for life. Thus, detection of EBNA-1 antibodies indicates past or recovering EBV infection (Smatti *et al*., 2018).

2.1.13. Laboratory diagnosis

Virus isolation can be done by culturing of EBV from oropharyngeal secretions, genital secretions, tumor specimens or peripheral blood, which cultivated with human umbilical cord lymphocytes, which are especially susceptible to EBV infection, and observed for 4 to 6 weeks for signs of cell transformation. Culturing EBV by the transformation assay is tedious and usually performed only for research studies (Jenson, 2011).

Serological diagnosis depends on detection of heterophile antibodies by their property to agglutinate cells from species different from those of the source serum (sheep, horse and beef red blood cell). EBV specific antibodies detection is done by detecting presence of antibodies to EBV early antigen (EA), IgM and IgG antibodies to VCA and antibodies to EBNA1 (Jenson, 2011).

2.1.13.1. Common immunological techniques used for detection of EBV specific antibodies

2.1.13.1.1. Immunofluresent assay

Classical method, gold standard and highly specific, staging of EBV infections possible with a single serum sample. It is generally performed with human EBV-transformed B-cell lines (Hess, 2004).

2.1.13.1.2. EIA, ELISA, or chemoluminescence with coated beads

Rapid, highly sensitive, suitable for automation, synthetic peptides as antigens less sensitive and less specific with a single serum sample (Hess, 2004).

2.1.13.1.3. Blot techniques (Western blot analysis / line blot assays)

It is confirmatory methods for screening assays, using viral lysates of EBV-transformed cells (De Paschale, 2012).

2.1.13.1.4. Advanced molecular diagnostic techniques

Quantitative real-time PCR for estimating viral load in different samples has been shown to be sensitive and reliable method in the diagnosis and management of EBV infections. It is a complementary tool to other serologic markers, in particular, for diagnosis of EBV acute infection and EBV silent reactivation (Smatti *et al.*, 2018).

2.1.13.1.5. Complement fixation reaction

Less sensitive, less specific and not widely used, staging of EBV infections not possible with a single serum sample (Hess, 2004).

2.1.13.1.6. IgG avidity

The IgG avidity test can assess the degree of IgG maturation. Avidity is low at the beginning of an acute infection, but increases when the immune response matures. Avidity can be measured using an EIA, IFA or immunoblotting (De Paschale, 2012).

2.1.14. Prevention and Control

EBV vaccine is not yet available for general use. The challenge is to assemble a group of individuals dedicated to its development and identify the resources needed. So that EBV vaccine can be given to all those who could benefit from it (Balfour, 2014).

Development of an EBV vaccine has been agonizingly slow because of difficulties in establishin g a suitable animal mode (Balfour, 2007).

2.1.15. Treatment

Numerous agents have been tried for the treatment to EBV, while anecdotal reports suggest that antiviral therapy (e.g. acyclovir, ganciclovir and vidarabine) might be effective in some cases of EBV, these agents inhibit the viral DNA polymerase and therefore inhibit replication of EBV in lytically infected cells that express the viral polymerase. Antiviral therapy is generally ineffective for this disease (Cohen, 2009).

2.2. Systemic Lupus Erythematous

Systemic lupus erythematosus (SLE) is an autoimmune disease with an incidence of 6–35 new cases per 100.000 per year and without clear pathogenesis. It is typically presents in women (90% of cases) in the reproductive age and characterized by polyclonal B cell activation and altered T cell function with the presence of multiple autoantibodies and impaired cell-mediated immunity (Draborg *et al*., 2012; Li *et al*., 2018).

There is strong epidemiologic evidence linking environmental factors with the development of SLE, including current cigarette smoking, crystalline silica exposure, alcohol consumption (decreased risk), UV lights, drugs, special chemistry substances and infections. Studies identifying modifiable environmental risk factors for the development of SLE are advancing our understanding of disease pathogenesis and could lead to strategies to prevent disease, in particular for those individuals at high risk (Barbhaiya and Costenbader, 2016).

The clinical criteria of disease involve dermatologic symptoms including a butterfly rash on the malar region of the face, discoid rash, photosensitivity, and oral or nasopharyngeal ulcers. Different hematologic disorders are also included: anemia, leucopenia, lymphocytopenia, and thrombocytopenia. In addition, presence of immunologic disorders including: antinuclear antibodies (ANAs), which are observed in 80–90% of SLE patients (Draborg *et al*., 2012).

Data from the UK primary care using the Clinical Practice Research Data link showed that the time from symptom onset to diagnosis of SLE has been reported to be approximately 2 years. Not all manifestations appear simultaneously and occasionally, a time interval of several months or years may exist between them. Musculoskeletal symptoms were most frequently (58.6%) recorded in the 5 year-period before SLE definitive diagnosis, and conversely, only few patients reported signs of active involvement of the kidneys (proteinuria or cellular casts) or other major organs (serositis, seizures, or psychosis) prior to SLE diagnosis. It has been reported that children, males

and patients with late-onset SLE (over the age of 50) have a longer time from first symptom to diagnosis than adult-onset SLE (Heinlen *et al*., 2007; Nightingale *et al.*, 2017).

One hundred sixteen autoantibodies were described in SLE patients. These include autoantibodies that target nuclear antigens, cytoplasmic antigens, cell membrane antigens, phospholipid-associated antigens, blood cells, endothelial cells, and nervous system antigens, plasma proteins, matrix proteins, and miscellaneous antigens. The mechanisms leading to the production and perpetuation of these aberrant autoimmune responses remain poorly understood (James *et al*., 2001; Sherer *et al*., 2004).

According to the current paradigm, SLE may be triggered in a genetically-susceptible individual by exposure to certain environmental risk factors and some studies showed that lupus heritability is approximately 44% which is lower than previously reported estimates (up to 66%) (Kuo *et al.*, 2015; Hedrich, 2017).

2.3. EBV infection in Systemic Lupus Erythematous

Though not much is known about the exact role of EBV in SLE pathogenesis and EBV is an excellent candidate to be involved in molecular mimicry in lupus. Infection with EBV results in the production of the viral protein Epstein-Barr virus nuclear antigen-1 (EBNA-1), antibodies against which cross-react with lupus-associated autoantigens. The immune response against EBV, and EBNA-1 in particular, differs among lupus patients and healthy controls, with controls maintaining a limited humoral response and failing to produce long-standing cross-reactive antibodies. The humoral immune response to EBNA-1 in susceptible individuals leads to the generation of cross-reactive antibodies. Through the process of epitope spreading, these cross-reactive antibodies target additional, non-cross reactive autoepitopes, spread to additional autoantigens, and become pathogenic, leading eventually to clinical lupus (Poole *et al*., 2006).

2.4. Previous Studies

Parks and her colleagues have a study (2005) in United States about the association of EBV with SLE in African Americans and Whites, and matched controls using ELISA. They found the anti-EBV-VCA was positive in 1% of African Americans among SLE patients and negative in controls (0%). Also infection rate of Whites was similar among cases and controls (1%) (Parks *et al.*, 2005).

Another study conducted by Chen and his colleagues (2005) in Taiwan, they evaluated of immunoglobulins antibodies against EBV capsid antigen in adult patients with lupus using immunofluorescence method. They found that 11.1% of SLE patients and 8.3% of controls were positive against IgM anti-EBV capsid Ag (Chen e*t al.*, 2005).

Han and others (2018) in China studied the relationship between EBV infection and type I interferon signature in patients with systemic lupus erythematosus using ELISA. They found anti-EBV capsid Ag (IgM) was 39.66% and 10.66% of serum specimens of cases and controls examined, respectively (Han *et al.*, 2018).

Also Li and others (2018) in China examined the association between EBV and SLE patients by conducting a systematic review and meta-analysis of case–control studies that detected the prevalence of EBV antibodies and the DNA-positive rate. They searched the MEDLINE and EMBASE databases, and there were eight studies that measured VCA IgM, with total of 665 patients and 648 controls. The overall percentage of those SLE positive with VCA IgM antibodies was higher than that for controls, (21.1%) and (8.3%) respectively (Li *et al*., 2018).

Another study carried by Tazi and others (2009) in Casablanca, Morocco determined the prevalence of antibodies to EBV in patients with SLE and healthy blood donors using ELISA; they found that the IgM against VCA was 9% in patients and 11% in controls (Tazi *et al.*, 2009).

A case-control study conducted by Us and her colleagues (2011) in Turkey to investigate the role of EBV and Herpes Simplex Virus in the etiology of Rheumatoid Arthritis and SLE. Their result about anti-EBV IgM against VCA was negative in all SLE patients and controls (0%) (Us *et al*., 2011).

Piroozmand and his colleagues have a cross-sectional study (2017) in Iran about the correlation between EBV infection and disease activity of SLE using real-time polymerase chain reaction method. Their result showed that; the EBV was positive in 67.5% of SLE patients with different disease activity (Piroozmand *et al*., 2017).

Another case-control study carried by Elsaied and others (2016) in Egypt to detect the association between serum nitric oxide and Epstein–Barr virus in patients with systemic lupus erythematosus, they didn't found any recent EBV infection among SLE patients and controls (0%) (Elsaied *et al.*, 2016).

CHAPTER III

MATERIALS AND METHODS

CHAPTER III

3. MATERIALS AND METHODS

3.1. Study design

This study was descriptive, case-control, hospital-based study.

3.2. Study area

The study was conducted at Military Teaching Hospital, Dr. Alnour Alaqeb clinic in Khartoum State.

3.3. Study duration

All work was done in the period from October 2019 to March 2021.

3.4. Study population

SLE patients, both males and females with age ranged from 20 to 70 years and healthy subjects matched in age and gender.

3.4.1. Inclusion criteria

Sudanese patients diagnosed with SLE with different age, sex and symptoms of disease such as joint pain, stiffness and swelling, chest pain and sometimes with skin lesions and rashes. Apparently healthy individuals were selected as controls with matching age and gender.

3.4.2. Exclusion criteria

Sudanese SLE patients diagnosed with other autoimmune disorders were excluded from this study.

3.5. Ethical considerations

Approval to conduct this study was obtained from Scientific Research Committee, College of Medical Laboratory Science, Sudan University of Science and Technology. The approval was taken from the Training Administration Department in the General Administration of Medical Services of the Military Hospital. Participants were informed about purpose of study and verbal consent was taken before collecting samples.

3.6. Sample size

Ninety two (n=92) participants were enrolled in this study and categorized as follow: 46 were patients with SLE and 46 were individuals without SLE with matching age and gender.

3.7. Data collection

The data were collected by direct interviewing checklist (appendix-1).

3.8. Laboratory processing

3.8.1. Collection of specimens

Non-probability convenience sampling technique was used in this study, in which 3ml venous

blood was drawn aseptically using syringe closed system into plain container. Specimens were allowed to clot and serum was separated by centrifugation at 5000 rpm for 5 minutes. Then sera were stored at -20℃ until performance of the ELISA technique.

3.8.2. Enzyme-Linked Immunosorbent Assay (ELISA)

Detection of anti- EBV–CA IgM was done by indirect ELISA which indicate to recent infection.

3.8.2.1. Procedure

The procedure was carried out according to guidance of manufacturing (PerkinElmer, USA) (appendix-2), in which 100 µL of the calibrators, positive control, and negative control and diluted patient sera were dispensed into designated wells of the 96-well microtiter plate.

After incubating the mixture at room temperature for 30 minutes, washing was done 3 times by the working wash buffer.

Then 100 µL of the enzyme conjugate was added in all wells and incubated for 30 minutes at room temperature, then the wells were washed 3 times using working wash buffer.

After that, 100 µL of chromogenic substrate was added into each well and incubated for 15 minutes at room temperature.

Finally, the reaction was stopped by adding 100 µL of sulphuric acid to all wells and the optical density was read spectrophotometrically using ELISA reader at wavelength of 450 nm (appendix-3).

3.8.2.2. Calculation

Results had been evaluated semi-quantitatively by dividing the OD values of each sample by obtained OD value of Cut off, according to the formula in the pamphlet (Sample O.D./ Cut off O.D.). To obtain cut off value was calculated as follow: OD of calibrator × Factor (f) printed on label of calibrator.

3.8.2.3. Interpretation of the results

Wells of samples with ratio greater than or equivalent to 1 were considered positive which indicate recent infection, while samples with ratio less than 0.9 were considered negative. Samples with ratio between 0.91-0.99 should be retested.

3.9. Data analysis

The data were analyzed and presented using Statistical Package for Social Science (SPSS) computer software version 20.0 for windows.

Frequencies were presented in form of tables and figures and significance of differences was determined using Chi-square test and statistical significance was set at *P-value*=0.05.

CHAPTER IV

RESULTS

CHAPTER IV

4. RESULTS

4.1. Distribution of SLE patients according to age and gender

A total of 46 blood specimens were collected from SLE patients with age ranged from 20 to 70 years with mean of 35.4 ± 11.7 S.D. The age was divided into 3 groups as following: 24 (52.18%) in age group 20-35 years, 17 (36.95%) in age between 36-51 years and 5 (10.87%) in age from 52 to 67 years. They were in different gender types, in which 39 (84.78%) were females and 7 (15.22%) were males (figure 4.1).

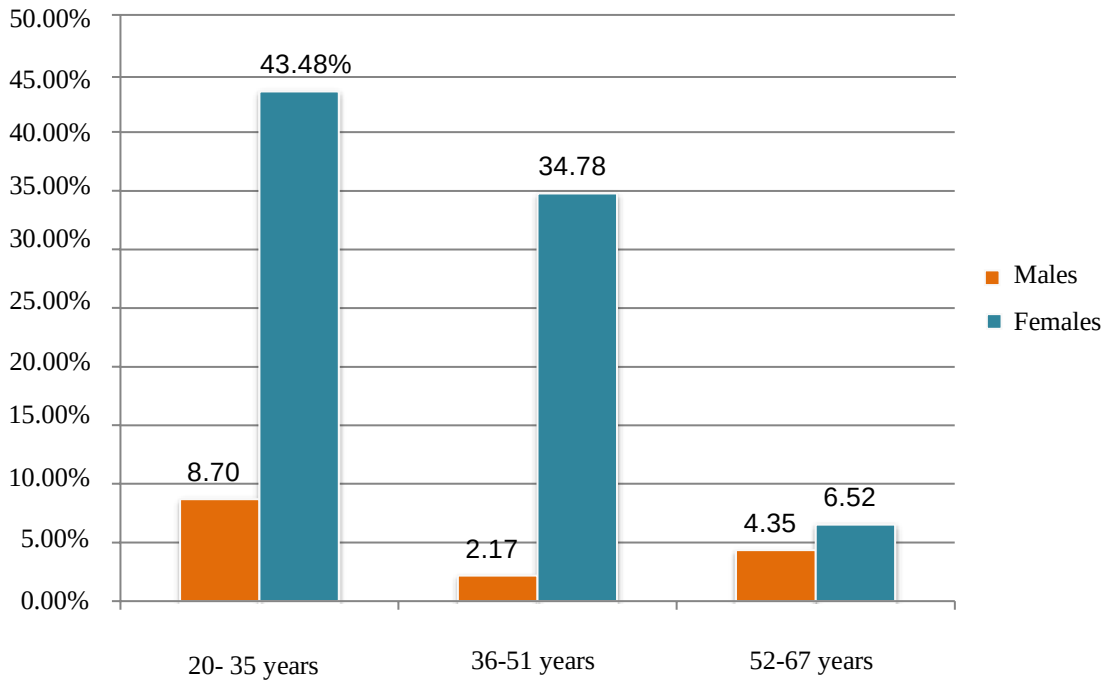

Figure 4.1 Distribution of SLE patients according to age and gender

4.2. Distribution of non-SLE individuals according to age and gender

A total of 46 non-SLE individuals were enrolled in this study and their age was ranged from 20 to 70 years with mean of 37.3 ± 11.7 S.D. The age was divided into 3 groups as following: 23 (50%) in age group 20-35 years, 15 (32.61%) in age between 36-51 years and 8 (17.39%) in age from 52 to 67 years.

Among these individuals the frequency of males was 23 (50%) while 23 (50%) was females (figure 4.2).

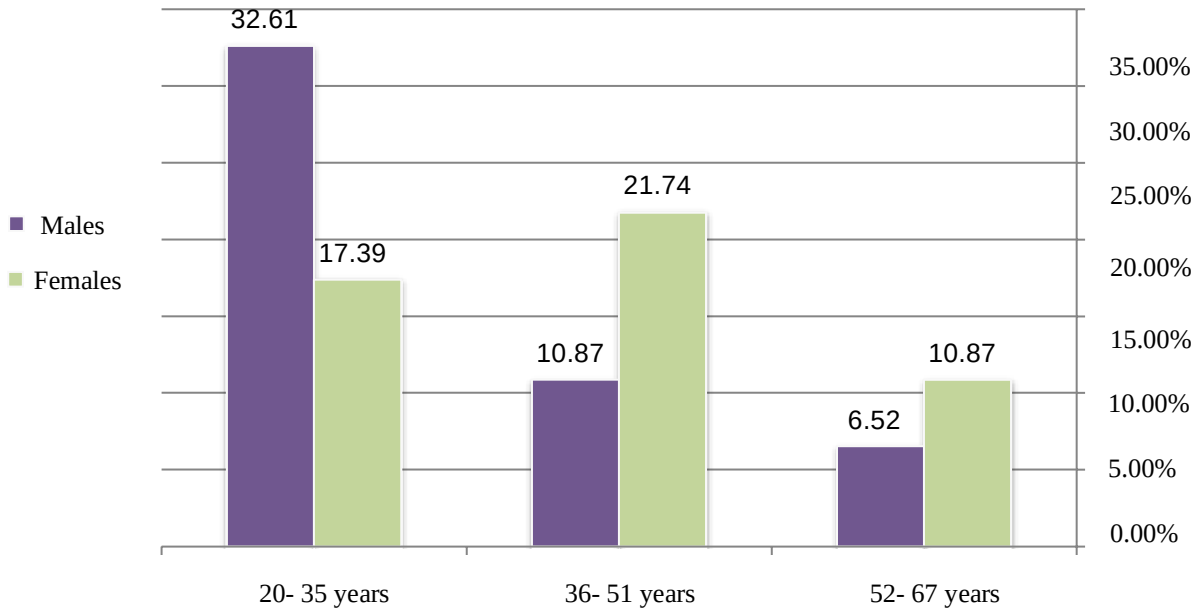

Figure 4.2: Distribution of non-SLE individuals according to age and gender

4.3. Frequency of anti-EBV–CA IgM antibodies among SLE patients

Out of 46 SLE patients, 15 (32.61%) were found to be positive for anti- EBV–CA IgM and 31 (67.39%) were negative against anti- EBV–CA IgM antibodies.

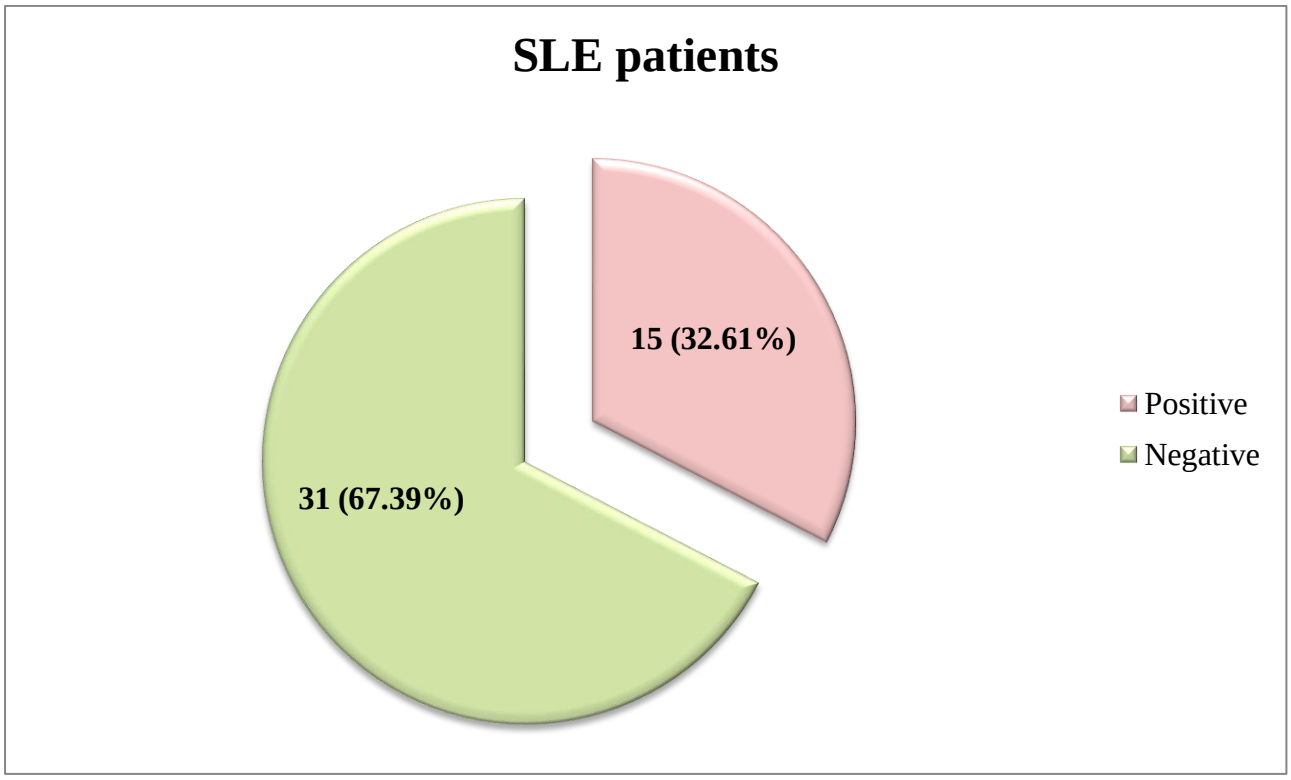

Figure 4.3: Frequency of anti-EBV-CA IgM antibodies among SLE patients

4.4. Frequency of anti-EBV–CA IgM antibodies among non-SLE individuals

Out of 46 non-SLE individuals, 7 (15.22%) were found to be positive for anti- EBV–CA IgM and 39 (84.78%) were negative against anti- EBV–CA IgM antibodies.

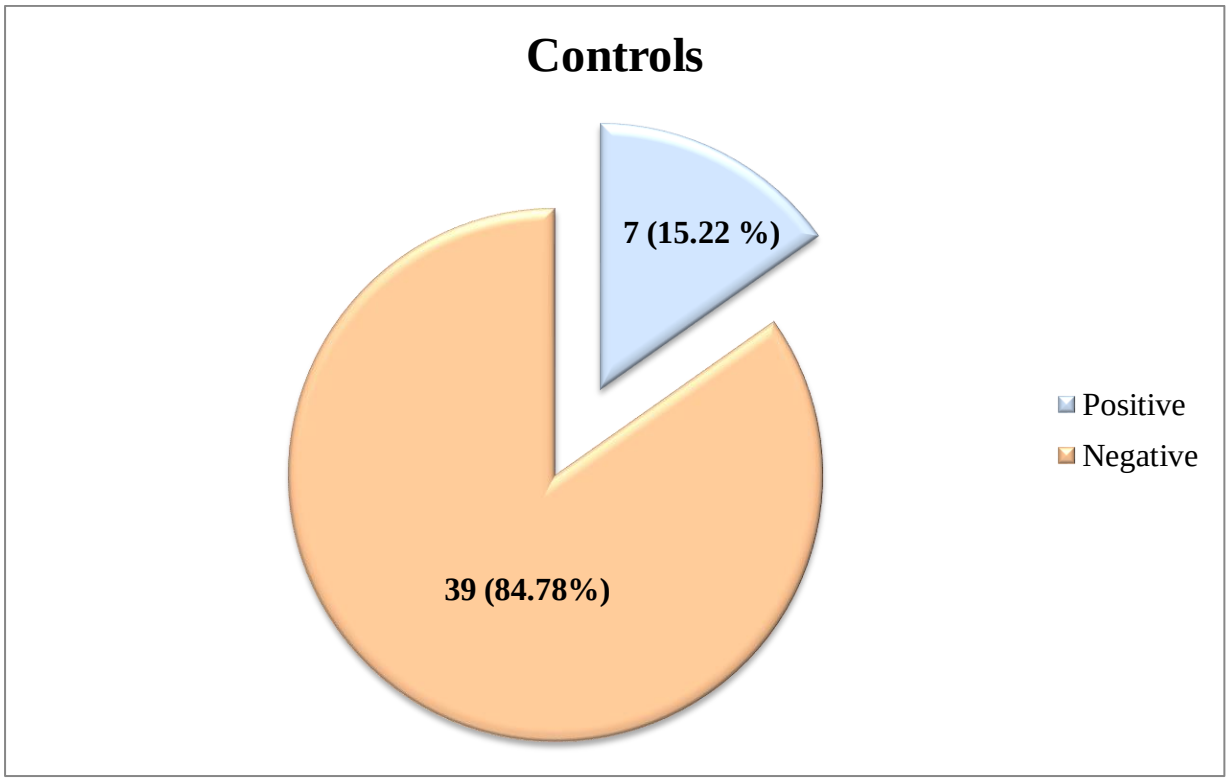

Figure 4.4: Frequency of anti- EBV–CA IgM antibodies among non-SLE individuals

4.5. The association between age and anti- EBV–CA IgM among SLE patients

Table 4.1 displayed that; the highest rate of positivity for anti-EBV (IgM) among SLE patients was 7 (15.22%) in age between 20 to 35 years then 6 (13.04%) in age group from 36 to 51 years and the lowest positivity in age ranged 52-67 years (2 (4.34%)). There was no significant association (*P*=0.857) between age and EBV infection.

Table 4.1: The association between age and anti- EBV–CA IgM among SLE patients

Age group/Years	Anti- EBV–CA IgM		Total	*P-value*
	Positive	Negative		
20-35 years	7 (15.22%)	17 (36.96%)	24 (52.18%)	0.857
36-51 years	6 (13.04%)	11 (23.91%)	17 (36.95%)	
52-67 years	2 (4.34%)	3 (6.52%)	5 (10.87%)	
Total	15(32.61%)	31 (67.39%)	46 (100%)	

4.6. The association between gender and anti- EBV–CA IgM among SLE patients

Tables 4.2 demonstrated that; there were 10 (21.74%) females and 5 (10.87%) males found positive to anti−EBV capsid Ag (IgM) among SLE patients with significant association (*P*=0.017).

Table 4.2: The association between gender and anti- EBV–CA IgM among SLE patients

Gender	Anti- EBV–CA IgM		Total	*P-value*
	Positive	Negative		
Males	5 (10.87%)	2 (4.35%)	7 (15.22%)	0.017
Females	10 (21.74%)	29 (63.04%)	39 (84.78%)	
Total	15 (32.61%)	31 (67.39%)	46 (100%)	

4.7. The association between age and anti- EBV–CA IgM among non-SLE individuals

Table 4.3 showed that; 6 (13.04%) in age group 20-35 years and 1 (2.17%) in age group 52-67 years were positive for anti-EBV-CA IgM among non-SLE individuals and no candidate was positive in age group from 36 to 51 years. There was insignificant association (*P*=0.089) between age and EBV infection.

Table 4.3: The association between age and anti- EBV–CA IgM among non-SLE individuals

Age group/Years	Anti- EBV–CA IgM		Total	*P-value*
	Positive	Negative		
20-35 years	6 (13.04%)	17 (36.96%)	23 (50%)	0.089
36-51 years	0 (0%)	15 (32.61%)	15 (32.61%)	
52-67 years	1 (2.17%)	7 (15.21%)	8 (17.39%)	
Total	7 (15.21%)	39 (84.78%)	46 (100%)	

4.8. The association between gender and the anti- EBV–CA IgM among non-SLE individuals

In this study; 6 (13.04%) males and 1 (2.17%) female were found positive to anti−EBV capsid Ag (IgM) among individuals without SLE and there was significant association (*P*=0.040) between them as displayed in table 4.4.

Table 4.4: The association between gender and anti- EBV–CA IgM among non-SLE individuals

Gender	Anti- EBV–CA IgM		Total	*P-value*
	Positive	Negative		
Males	6 (13.04%)	17 (36.96%)	23 (50%)	0.040
Females	1 (2.17%)	22 (47.82%)	23 (50%)	
Total	7 (15.21%)	39 (84.78%)	46 (100%)	

CHAPTER V

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

CHAPTER V

5. DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1. Discussion

This study found that 15/46 (32.61%) SLE patients and 7/46 (15.21%) individuals without SLE were positive for anti- EBV–CA IgM, indicating recent infection which was near to those obtained by Han *et al*. (2018) in China, in which the positivity rates were 39.66% and 10.53% in SLE patients and healthy controls, respectively.

The rate of seropositivity in SLE patients and individuals without SLE revealed by this study was higher than that reported by Chen e*t al.* (2005) in Taiwan, in which there were 4/36 (11.1%) SLE patients and 3/36 (8.3%) healthy individuals were positive for anti- EBV–CA IgM and Tazi *et al.* (2009) in Casablanca, Morocco found that 4/44 (9%) SLE patients and 5/44 (11%) healthy blood donors were positive for IgM antibodies against EBV-CA.

Also Parks *et al*. (2005) in United States; they found that 2/144 (1%) of African Americans and 1/86 (1%) of Whites among SLE patients were positive for anti-EBV – CA IgM. Also among individuals without SLE, 0/72 (0%) of African Americans and 1/204 (1%) of Whites were found positive for anti-EBV – CA IgM.

The present study was contradictory to Us T *et al*. (2011) in Turkey who found IgM antibodies against CA were negative in all of 50 SLE patients and 50 healthy blood donors (0%), and the same result was obtained by Elsaied *et al*. (2016) in Egypt, in which 0/38 (0%) of SLE patients and 0/32 (0%) of healthy controls were negative for anti- EBV–CA IgM.

This findings were lower than those obtained by Piroozmand *et al*. (2017) in Iran, their study showed that the EBV was positive in 27/40 (67.5%) of SLE patients with different disease activity.

Reasons for these variations between results could be due to difference in geographic regions, level of hygiene, educational and social status, endemicity of virus, different lifetime exposures of the participants to EBV and different techniques and methods used for virus detection.

In this study, the higher rate of infection was in age from 20 to 35 years among both SLE and non-SLE individuals, which was 15.22% and 13.04% respectively. However, there was no significant association between the age and EBV infection; this was closed to the results obtained by Chen *et al*. (2005) in Taiwan, the infection rate was high in age 20 and above and Tazi *et al.* (2009) in Morocco in which the age was ranged from 19 to 55.

Furthermore, noted that the seroprevalence of EBV infection was significantly higher in females (21.74%) compared to males (10.87%) among SLE patients, this was agreed with results obtained by Piroozmand *et al*. (2017) in Iran, in which the rate of EBV infection was 92.5% in females and 7.5% in males. In contrary, among non-SLE individuals the seroprevalence of EBV infection was high in males (13.04%) compared to females (2.17%).

Among both groups there were significant association between gender and EBV infection rate. This variation of infectivity between males and females maybe related to different distribution of gender between SLE patients and non-SLE individuals, so it is need further studies with inclusion of more sample size and even distribution of gender to roll out the causes underline this variation.

5.2. Conclusion

The findings of this study were concluded that; EBV was detected in few cases of SLE and individuals without SLE.

There was significant association between the gender and EBV infection among SLE patients and non-SLE individuals.

There was no significant association between the age and EBV infection among SLE patients and non-SLE individuals.

5.3. Recommendations

-Specific and sensitive EBV detection techniques should be used e.g. Polymerase Chain Reaction (PCR) and Real time PCR.

-Detection of two types of antibodies IgM and IgG against specific viral antigens should be carried on future studies.

-Further studies should be carried out with increase the sample size for evaluation of specific antigens, viral load or alleles variant to determine the association between EBV and SLE as causal relationship.

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ADDPENDICES

APPENDIX-1

Questionnaire

Sudan University of Science and Technology

College of Graduated Studies

Sero-Detection of Epstein-Barr Virus among Systemic Lupus Erythematosus patients in Khartoum State

Patient No.: .....

Gender:

Male ( ) Female ( )

Age:

20 - 35 years ( )

36 - 51 years ( )

52 - 70 years ( )

The Result:

Anti- EBV–CA IgM:

+ve ( ) -ve ( )

APPENDIX-2

EBV-VCA-M-96T

PerkinElmer
For the Better

MICROWELL ELISA

EBV-VCA IgM
Catalog No. 10202

(96 Tests)

SUMMARY OF ASSAY PROCEDURE

Step	(20-25°C Room temp.)	Volume	Incubation time
1	Sample dilution 1:21 = 10 µL / 200 µL		
2	Diluted samples, controls & calibrators	100 µL	30 minutes
3	Washing buffer (3 times)	350 µL	
4	Enzyme conjugate	100 µL	30 minutes
5	Washing buffer (3 times)	350 µL	
6	TMB Chromogenic Substrate	100 µL	15 minutes
7	Stop solution	100 µL	
8	Reading OD 450 nm		

NAME AND INTENDED USE

The Epstein-Barr Virus-Viral Capsid Antigen (EBV-VCA) IgM Enzyme-linked Immunosorbent Assay (ELISA), is intended for the detection of IgM antibody to Epstein-Barr virus in human serum.

SUMMARY AND EXPLANATION OF THE TEST

Detection of the Epstein-Barr virus was first described in 1964 by Epstein, Achong, and Barr using electron microscopic studies of cultured lymphoblasts derived from patients with Burkitt's lymphoma. [1]
EBV is classified as a member of the herpes-virus family based upon it's characteristic morphology[2,3].
EBV infection may demonstrate a wide spectrum of clinical symptoms. The majority of primary EBV infections are transmitted via saliva, occur during childhood, and are subclinical. In the U.S., 50% of the population demonstrate EBV antibodies before the age of 5 years; 80% by adulthood. Transfusion-associated EBV infections have also been reported[2]. In young adults, EBV infection may be clinically manifested as Infectious Mononucleosis (IM) with typical symptoms of sore throat, fever, and lymphadenopathy.[3] College students and military personnel are often cited as a high morbidity incidence population for IM[2].
Following primary EBV infection, it is postulated that the B lymphocyte may continue to harbor the EBV genome and establish a latent infection that may extend through life.[4] Reactivation of EBV infection or enhanced EBV activation has been documented in immunodeficient or immunosuppressed patients, i.e., organ transplant patients, individuals with malignancies, pregnant women, and persons of advanced age.[4]
Epstein-Barr virus has also been associated in the pathogenesis of two human cancers, Burkitt's lymphoma and nasopharyngeal carcinoma. Documentation by means of DNA hybridization studies demonstrates the presence of the EBV genome on biopsy specimens taken from individuals with these carcinomas.[3]
Burkitt's lymphoma is primarily observed in Sub-Sahara Africa, especially in African children, and in New Guinea. Malarial infections are usually diagnosed in Burkitt's lymphoma patients and are suggested to be a co-factor[5,6]. Nasopharyngeal carcinoma is observed in Asia, most notably in Southern China, and may have genetic or environmental influences as the co-factor[5,6].
In the last two decades, serological methods have progressed from testing for the presence of non-specific heterophile antibodies to measuring levels of IgG or IgM formed against subunits of EBV antigen complexes. One of the best indicators of active EBV infection is antibody to viral capsid antigens, structural proteins necessary for replication of the virus.[7] Viral capsid antigens are present in every cell infected with EBV. The IgM response to VCA is among the earliest detectable humoral immune responses, usually present at the onset of the disease and peaking within four to six weeks. VCA-IgM levels are also transient, declining rapidly and usually becoming undetectable within two to three months from onset of clinical symptoms.[8]

PRINCIPLE OF THE TEST

Purified EBV-VCA antigen is coated on the surface of microwells. Diluted patient serum is added to wells, and the EBV-VCA IgM specific antibody, if present, binds to the antigen. All unbound materials are washed away. After adding enzyme conjugate, it binds to the antibody-antigen complex. Excess enzyme conjugate is washed off, and TMB Chromogenic substrate is added. The enzyme conjugate catalytic reaction is stopped at a specific time. The intensity of the color generated is proportional to the amount of IgM specific antibody in the sample. The results are read by a microwell reader compared in a parallel manner with calibrator and controls.

MATERIALS PROVIDED

1. Microwell strips: EBV-VCA antigen coated wells. (12 x 8 wells)
2. Absorbent solution: Yellow Color Solution. 1 vial (22 mL)
3. Calibrator: Factor value (f) stated on label. Red Cap. 1 vial (150 µL)
4. Negative Control: Range stated on label. Natural Cap.1 vial (150 µL)
5. Positive Control: Range stated on label. Brown Cap. 1 vial (150 µL)
6. Washing Concentrate 20x(H).1 bottle (50 mL)
7. Enzyme Conjugate: Red color solution.1 vial (12 mL)
8. TMB Chromogenic Substrate: Amber bottle.1 vial (12 mL)
9. Stop Solution.1 vial (12 mL)

STORAGE AND STABILITY

1. Store the kit at 2 - 8 °C.
2. Always keep microwells tightly sealed in pouch with desiccants. We recommend you use up all wells within 4 weeks after initial opening of the pouch.
3. The reagents are stable until expiration of the kit.
4. Do not expose test reagents to heat, sun or strong light during storage or usage.

WARNINGS AND PRECAUTIONS

1. Potential biohazardous materials:The calibrator and controls contain human source components which have been tested and found nonreactive for hepatitis B surface antigen as well as HIV antibody with FDA licensed reagents. However, as there is no test method that can offer complete assurance that HIV, Hepatitis B virus or other infectious agents are absent, these reagents should be handled at the Biosafety Level 2, as recommended in the Centers for Disease Control/National Institutes of Health manual, "Biosafety in Microbiological and Biomedical Laboratories." 1984
2. Do not pipette by mouth. Do not smoke, eat, or drink in the areas in which specimens or kit reagents are handled.
3. The components in this kit are intended for use as a integral unit. The components of different lots should not be mixed.
4. This product contains components preserved with sodium azide. Sodium azide may react with lead and copper plumbing to form explosive metal azide. On disposal, flush with a large volume of water.

SPECIMEN COLLECTION AND HANDLING

1. Collect blood specimens and separate the serum.
2. Specimens may be refrigerated at 2 - 8°C for up to seven days or frozen for up to six months. Avoid repetitive freezing and thawing of serum sample.

PREPARATION FOR ASSAY

1. Prepare 1x washing buffer. Prepare washing buffer by adding distilled or deionized water to 20x(H) wash concentrate to a final volume of 1 liter.
2. Bring all specimens and kit reagents to room temperature (20-25 °C) and gently mix.

ASSAY PROCEDURE

1.Collect blood specimens and separate the serum.
2.Specimens may be refrigerated at 2 - 8 °C for up to seven days or frozen for up to six months. Avoid repetitive freezing and thawing of serum sample.
3.Place the desired number of coated strips into the holder.
4.Prepare 1:20 dilutions by adding 10 µL of samples, negative control, positive control, and calibrator to 200 µL of absorbent solution. Mix well.
5.Dispense 100 µ L of diluted sera, calibrator, and controls into the appropriate wells. For the reagent blank, dispense 100 µ L Absorbent solution in 1A well position. Tap the holder to remove air bubbles from the liquid and mix well. Incubate for 30 minutes at room temperature.
6.Remove liquid from all wells. Repeat washing three times with washing buffer.
7.Dispense 100 µL of enzyme conjugate to each well and incubate for 30 minutes at room temperature.
8.Remove enzyme conjugate from all wells. Repeat washing three times with washing buffer.
9.Dispense 100 µL of TMB Chromogenic Substrate to each well and incubate for 15 minutes at room temperature.
10.Add 100 µL of Stop solution to stop reaction.
Make sure there are no air bubbles in each well before reading.
11.Read O.D. at 450 nm with a microwell reader.

CALCULATION OF RESULTS

1.To obtain Cut off OD value: Multiply the OD of Calibrator by Factor(f) printed on label of Calibrator.
2.Calculate the IgM Index of each determination by dividing the OD values of each sample by obtained OD value of Cut off.

For example:

- If Factor (f) value on label = 0.4
- This factor (f) is a variable. It is specific for a lot manufactured and printed on label of Calibrator.
- Obtained Calibrator O.D. = 1.100
- Cut-off O.D. = 1.100 x 0.4 = 0.44 (By definition IgM Index = 1)
- Patient sample O.D. = 0.580
- IgM Index = 0.580 / 0.44 = 1.32 (Positive result)
- Patient sample O.D. = 0.320
- IgM Index = 0.320 / 0.44 = 0.73 (Negative result)

QUALITY CONTROL

The test run may be considered valid provided the following criteria are met:

1.The O.D. value of the reagent blank against air from a microwell reader should be less than 0.150.
2.If the O.D. value of the Calibrator is lower than 0.250, the test is not valid and must be repeated.
3.The EBV-VCA IgM Index for Negative and Positive Control should be in the range stated on the labels.

INTERPRETATION

Negative: EBV-VCA IgM Index of 0.90 or less are seronegative for IgM antibody to EBV-VCA virus.

Equivocal: EBV-VCA IgM Index of 0.91 - 0.99 are equivocal. Sample should be retested.

Positive: EBV-VCA IgM Index of 1.00 or greater are seropositive. Indicative of current of recent infection.

LIMITATIONS OF THE PROCEDURE

1.As with other serological assays, the results of these assays should be used in conjunction with information available from clinical evaluation and other diagnostic procedures.
2.The absence of detectable IgM antibody does not rule out the possibility of recent or current infection. A second specimen 5~7 days later should be repeated.
3.The absorbent solution used as sample diluent in this test is to prevent the interferences of specific IgG and rheumatoid factor (RF). However RF larger than 400 IU/mL may interfere with the test in the presence of high specific IgG.
4.A positive EBV IgM result is generally considered diagnostic for acute IM. To verify the diagnosis, however, it is recommended that the specimen be tested for other EBV antibodies, such as EA-D or EBNA IgG and EBNA IgM to determine predominant antibody.
5.For professional use only.

PERFORMANCE CHARACTERISTICS

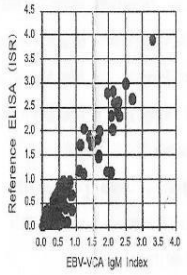
Sensitivity, Specificity, and Accuracy

A total of 124 random samples from different sources were assayed with ELISA EBV-VCA IgM test and with a commercially available ELISA test kit.

		Reference ELISA		
		N	P	Total
	N	100 (D)	0 (B)	100
ELISA	P	0(C)	24(A)	24
EBV-VCA IgM	Total	100	24	124

Relative Sensitivity = A / (A+B) = 24 / 24 + 0 = 100%
Relative Specificity = D / (C+D) = 100 / 100 + 0 = 100%
Relative Accuracy = (A+D) / (A+B+C+D) = 124 / 124 = 100%

The correlation of quantitative values between two comparison methods was summarized:



Cross-Reactivity:
A study was performed to determine the cross-reactivity of EBV-VCA IgM with other member of the HSV family and other IgM antibodies. A total of 44 samples negative for EBV-VCA IgM but positive for IgM for CMV (6), HSV 1 (4), HSV 2 (4), Rubella (10), Toxo (3), RF (7), and ANA IgG (10) by other commercial available kits were assayed. All 44 samples give negative results for EBV-VCA IgM. It indicates an absence of cross-reactivity of the ELISA EBV-VCA IgM with other members of HSV family and other IgM antibodies.

Precision:
The mean, SD, and % CV were calculated for inter- and intra-assay.

Intra-Assay	n	Index Mean	SD	% CV
Serum 1	8	0.185	0.0141	7.65
Serum 2	8	1.093	0.0200	1.82
Serum 3	8	1.479	0.0362	2.40
Serum 4	8	2.017	0.1356	6.70

Inter-Assay	n	Index Mean	SD	% CV
Serum 1	8	0.184	0.015	8.17
Serum 2	8	1.074	0.091	8.46
Serum 3	8	1.506	0.116	7.70
Serum 4	8	2.197	0.188	8.55

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(For Export Only)

APPENDIX-3

Microtiter plate