

# CHAPTER ONE

## 1. INTRODUCTION

### 1.1 Bakgraund

Epstein- Barr virus (EBV) is a DNA virus belong to the human gamma-herpes virus that infects and persists in more than 90 % of the world adult population clinically manifesting either as an asymptomatic infection or as acute infectious mononucleosis(IM) (Daud *et al.*, 2015). African children are infected early in life and nearly almost have sero-converted by 3 years, whilst in affluent countries, primary infection is belated until young adult life (Rasti *et al.*, 2005).

EBV has the outstanding property like other herpes viruses, it has the ability to enter a latent state following primary infection of their natural host and be reactivated aftertime, their frequent reactivation in immunosuppressed patients with impaired cellular immunity can causes serious health complications (Butal, 2010). The reactivation of the virus depend on a balance between viral latency, viral replication, and host immune responses (Donati *et al.*, 2006).

Malaria infection profoundly affects the B cell compartment, by stimulating polyclonal activation and hyper-gamma globulinemia. Acute malarial infection has a role in the impairment of the EBV-specific immune responses with the consequent increase in the number of EBV carrying B cells in the circulation (Bakhshi ,2007).

Both EBV infection and intense exposure to *Plasmodium falciparum* malaria (holoendemic malaria) are recognized as a cofactors in the pathogenesis of endemic Burkitt's lymphoma (eBL), which is the most

common pediatric cancer in equatorial Africa, accounting for up to 74% of childhood malignant disorders (Chêne *et al.*, 2007).

The effect of malarial infection in EBV reactivation and its contribution in the pathogenesis of eBL is not entirely understood (Rasti *et al.*, 2005). EBV is the etiological agent of acute infectious mononucleosis (IM), nasopharyngeal carcinoma (NPC), burkitt's lymphoma (BL), Hodgkin and non-hodgkin lymphomas (NHL), other lymphoproliferative disorders in immunodeficient individuals and gastric carcinoma (GC) ((Butal, 2010).

Several recent studies in many African countries reflect the role of *P. falciparum* malaria in reactivation of EBV (Rasti *et al.*, 2005; Donati *et al.*, 2006).

## **1.2 Rationale**

Over recent years, the concept of polymicrobial diseases has gained an increased attention in the recent past, due to the overlapping of different disease in same endemic areas (Chêne, 2009).

*P. falciparum* malaria is known to interfere with EBV biology and EBV-specific immunity and can induce EBV reactivation (Daud *et al.*, 2015).

Sudan has a history of frequent and devastating malaria epidemics in the low and unstable seasonal transmission areas in the Northern, River Nile, Khartoum, White Nile, Geziera, Kassala, Gedarif, Red Sea and North Kordofan, especially in urban areas. *P. falciparum* is responsible for more than 95% of malaria cases in Sudan. However, an increase in *P. vivax* cases has been noticed in the last years (Ministry of health, 2013).

Most available literature from Sudan dealt with BL without referring to specific investigation of etiologic factors. In a single study conducted by

Osman *et al.*, (2014), childhood lymphomas were predominantly BL and the percent of eBL was 7%.

In Sudan there is no previous studies and published data concerning malaria and EBV co-infections. This study was conducted to shine a spotlight on this topic.

### **1.3 Objectives**

#### **1.3.1 General objective**

To determine infection of Epstein-Barr virus in children with *Plasmodium falciparum* malaria.

#### **1.3.2 Specific objective**

- 1) To detect EBV DNA in plasma specimens of children with or without *P. falciparum* malaria infection.
- 2) To compare presence of EBV DNA in the two populations, with or without *P. falciparum* malaria.

## **CHAPTER TWO**

### **2. Literature review**

#### **2.1 Epstein-Barr Virus (EBV)**

##### **2.1.1 History of EBV**

EBV is in some respects the most sinister herpesvirus, for its association with malignant disease is now well established. In 1958, Burkitt described a tumor in African children that occurred in areas with a high prevalence of malaria. He thought that it might be caused by an infectious agent spread by mosquitoes (Collier *et al.*, 2006).

The mosquito theory was wrong, but 6 years later Epstein and his colleagues discovered a herpesvirus in cultures of the tumor cells. In 1966, American workers showed the association of this virus both with infectious mononucleosis (IM) and with another form of cancer, nasopharyngeal carcinoma (NPC), occurring mostly in southern China. In addition to its association with these three syndromes, EBV causes B-cell lymphomas in immunodeficient patients (Collier *et al.*, 2006).

##### **2.1.2 Viral morphology and genomic organization of EBV**

Characteristically, all of the herpesviruses cannot readily be differentiated by morphology in the electron microscope, because they all have similar appearances (Cornelissen *et al.*, 2013).

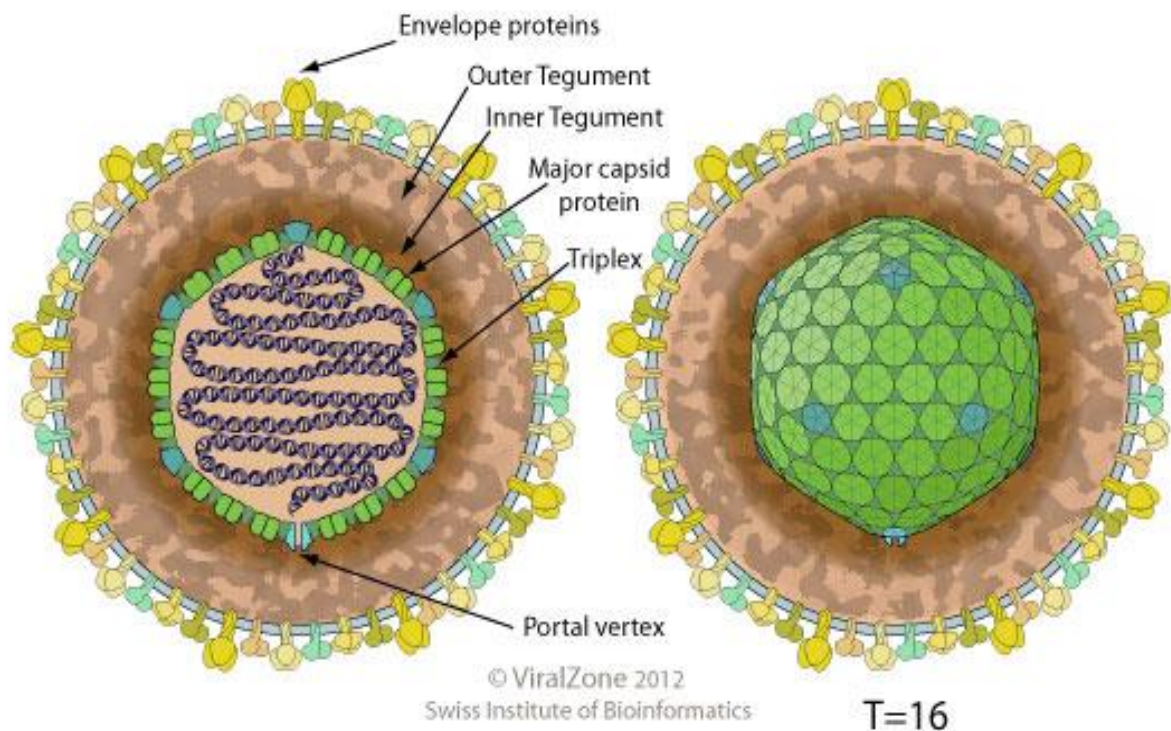
###### **2.1.2.1 Virion Structure**

Herpesvirus particles (virions) are spherical and have an approximate diameter of 200 nm (**Fig. 2.1**). The core is occupied by the virus genome, and surrounded by the icosahedral capsid of diameter 125–130 nm. It is fashioned from 161 protein capsomeres, which are contributed by 150 hexons and 11 pentons, plus the portal in the 12<sup>th</sup> pentonal position. The portal consists of 12 copies of the portal protein and forms the vertex

through which DNA enters and leaves the capsid. The capsomeres in the capsid shell are joined together via complexes known as triplexes (Davison, 2010).

The capsid is embedded in the tegument (Davison, 2010). The tegument (the space between capsid and envelope) is filled with proteins, and enzymes needed for replication and prepares them to do so (Ebell, 2004).

The nucleocapsid (capsid and the genome) are enclosed by an envelope containing glycoproteins and it is derived from the outer nuclear membrane of the host cell (Parija, 2009).



**Fig. 2.1: Epstein-Barr Virus morphology.** Enveloped, spherical to pleomorphic, 150-200 nm in diameter, icosahedral symmetry. Capsid consists of 162 capsomers joined together via complexes known as triplexes and is surrounded by an amorphous tegument. portal protein forms the vertex through which DNA leaves the capsid. Glycoproteins complexes are embedded in the lipid envelope. linear, dsDNA genome. Adopted from: [http://viralzone.expasy.org/all\\_by\\_species/185.html](http://viralzone.expasy.org/all_by_species/185.html)

### **2.1.2.2 Taxonomy and Genome Structure**

EBV; also known as *Human herpesvirus 4* (HHV-4), is a member of the genus *Lymphocryptovirus*, which belongs to the lymphotropic subfamily *Gammaherpesvirinae* of the family *Herpesviridae* (Young, 2010). There are two types of the virus, A and B (also known as types 1 and 2), Based on sequence variation in the EBV nuclear antigens 2 and 3 (EBNA 2 and 3) (Daud *et al.*, 2015).

EBV is about 170 kb, enveloped, and double-stranded DNA virus with a single linear genome, which is circularized after infection and maintained as an extrachromosomal episome. The genome encodes about 80 different proteins that are involved in both the latent and lytic phases of cycle (Daud *et al.*, 2015).

### **2.1.3 Viral Antigens**

EBV antigens are divided into three classes, based on the phase of the viral life cycle in which they are expressed (Butal, 2010).

#### **2.1.3.1 Latent phase antigens**

These antigens are synthesized by latently infected cells, include the Epstein-Barr viral nuclear antigens (EBNAs) found in the nuclei of infected cells and identified as six separate proteins (EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C; and leader protein, also called EBNA1–6) and the latent membrane proteins (LMPs) (LMP1, LMP2A and LMP2B) (Crawford, 2004).

Their expression reveals the presence of EBV genome. Only EBNA1, needed to maintain the viral DNA episomes, is invariably expressed. LMP1 mimics an activated growth factor receptor (Butal, 2010).

### **2.1.3.2 Early antigens**

These are nonstructural proteins whose synthesis is not dependent on viral DNA replication. The expression of early antigens denote the onset of productive viral replication (Butal, 2010).

### **2.1.3.3 Late antigens**

Late antigens are the structural components of the viral capsid (viral capsid antigen VCA) and viral envelope (glycoproteins) (Butal, 2010), the major antigen of this sort is gp340/220 (Collier *et al.*, 2006), which induces neutralizing antibody, and these antigens produced abundantly in cells undergoing productive viral infection (Butal, 2010).

### **2.1.4 Other Properties of EBV**

EBV is relatively fragile, sensitive to the action of ether and bile salts, and outside human body fluids cannot survive for a longer period (Parija, 2009).

EBV is unlike the other herpes viruses, it can be cultured easily only in lymphoblastoid cell lines derived from B lymphocytes of humans and higher primates (Drew, 2010).

EBV induces no cytopathic effect like those caused by other herpesviruses, although its ability to cause inflammatory disease shows its capacity for directly or indirectly damaging cells *in vivo* (Collier *et al.*, 2006).

### **2.1.5 Epidemiology of EBV**

EBV is common in all parts of the world, with over 90% of adults being seropositive. In developing poor communities, EBV is acquired early in life more than 90% of children are infected by age 6years when it causes mainly in apparent infections (Butal, 2010).



In industrialized countries, more than 50% of EBV infections are delayed until late adolescence and young adulthood. In almost half of cases, the infection is manifested by infectious mononucleosis (IM) (Butal, 2010).

Spreading of infection is associated with close personal contact, so it is not surprising that the age at first infection varies according to living conditions. Infection occurs at an earlier age in the presence of poor hygiene and crowded living conditions; but in developing countries and underprivileged societies infection rates in young children are higher (Junker, 2005).

International Agency for Research on Cancer (IARC) classify EBV as a group 1 carcinogen and considered to have direct role in the etiology of endemic form of BL, GC, NPC, and a subclass of Hodgkin lymphoma (IARC, 1997).

#### **2.1.6 Transmission of EBV**

The infection is transmitted by close oral contact or by sharing the glasses, cups and toothbrushes (Parija, 2009). The transmission by blood or bone marrow transfusions are rare events, it is of low contagiousness (Ananthanarayan and Paniker., 2006). Most cases of IM are contracted after repeated contact between susceptible persons and those asymptomatically shedding the virus (Drew, 2010).

The sexual transmission is a likely route of EBV transmission as well as raising the possibility of neonatal infection and breast milk is an alternative source of infectious virus for infants (Daud *et al.*, 2015).

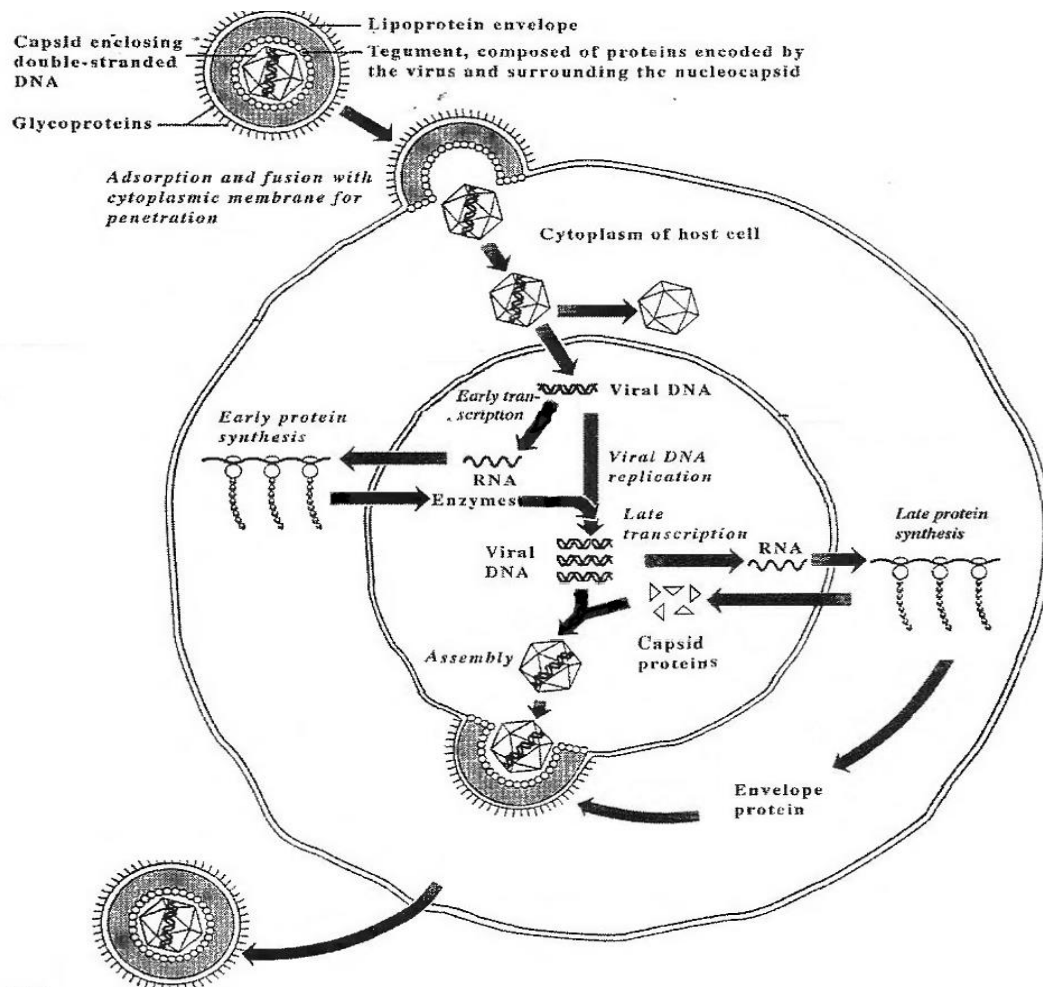
### **2.1.7 Replication of EBV (Fig. 2.2)**

During B cell entry, EBV gp350/220 binds to complement receptor 2 (CR2/CD21) concentrating virus to the B cell surface, but this interaction does not activate membrane fusion or virus entry (Sathiyamoorthy *et al.*, 2014).

The g42 protein forms stable, high affinity complexes, and binds to human leukocyte antigen class II which triggering receptor for EBV entry into B cells (Sathiyamoorthy *et al.*, 2014).

The viral capsid is transported to a nuclear pore, at which point the linear DNA is released and enters the nucleus of the cell where most events of transcription, viral DNA replication and capsid assembly occur. The virus induces a shutdown of host protein and nucleic acid synthesis (Collier *et al.*, 2006).

Newly synthesized envelope proteins accumulate in patches on the nuclear membrane, and nucleocapsids that have been assembled in the nucleus acquire their envelopes by budding through these patches, the completed virus is transported by a vacuole to the surface of the cell, The end result of this productive, lytic cycle is cell death because most cellular synthetic pathways are effectively turned off during viral replication (Cornelissen *et al.*, 2013).



**Fig. 2.2: Replication of Epstein-Barr Virus.** EBV adsorb to host cell receptors, the viral envelope fusion with the cell's plasma membrane then depositing the nucleocapsid and tegument proteins in the cytosol. The viral capsid is transported to a nuclear pore, DNA is released and enters the nucleus, initiation of viral DNA replication, intermediate and late genes are transcribed, Newly synthesized envelope proteins accumulate in patches on the nuclear membrane, and nucleocapsids that have been assembled in the nucleus acquire their envelopes by budding through these patches, the completed virus is transported by a vacuole to the surface of the cell and the end result of this productive, lytic cycle is cell death.

Adopted from: <https://microbitos.files.wordpress.com/2012/04/virus-epstein-barr-presentacion-pdf.pdf>

## **2.1.8 Life cycle**

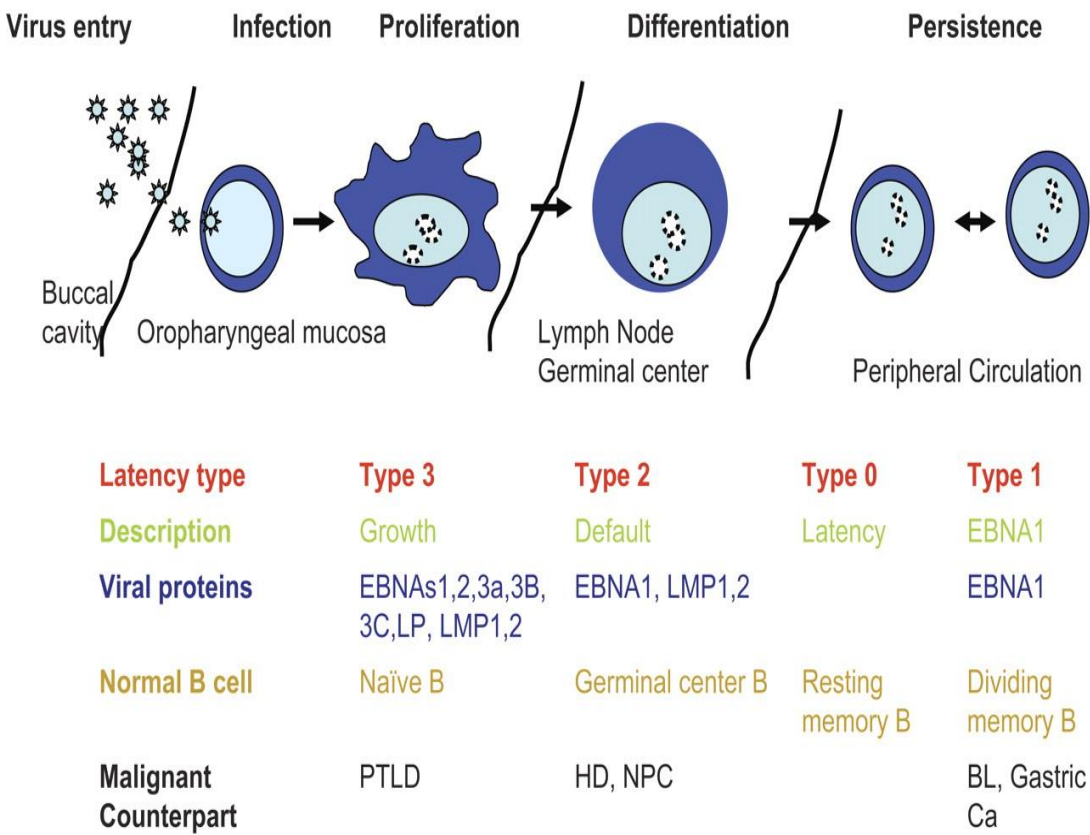
### **2.1.8.1 Establishment and maintenance of EBV latency**

EBV establishes latency exclusively in memory B cells. A model of EBV persistence (**Fig. 2.3**) links the establishment of EBV latency to early B cell development and emphasizes the intersection of EBV with germinal center (Chêne, 2009).

EBV spreads via the salivary tract to infect epithelial cells of the oropharynx, and possibly tonsillar lymphocytes, where the virus initiate lytic replication and releases progeny virions that in turn infect resting naïve B-cells in the underlying lymphoid tissues. After that EBV switches on the growth program (latency III) and expressed the latent proteins (LMFs, EBNAs and Epstein-Barr Virus encoded mRNA (EBERs) ). These latent proteins subsequently induce the resting naïve B-cells into an activated lymphoblast, that travel to the follicles where they initiate germinal center reactions and establish the default transcription program (latency II). During the latency II program the virus switches on survival signals by expressing LMP1, which protects the cell from apoptosis and drives class switch recombination, and LMP2A that drives somatic hyper-mutation (Daud *et al.*, 2015).

The cell may exit the germinal center and enter the memory compartment. EBV switches to the latency program (latency 0) with latent gene expression restricted to EBERs. Memory cells circulate in the blood and in the secondary lymphoid organs. During homeostasis cell division, EBV expresses EBNA1 alone (latency1). Latently infected cells can re-enter the Waldeyer's ring where they can divided upon encounter of bystander T helper cells. Some are re-introduced in the circulation and some

differentiate into plasma cells, migrate to the lymphoepithelium and release virions insuring a persistent infection (Chêne, 2009).



**Fig. 2.3: Epstein-Barr Virus latent life cycle.** Virus enters though mucosal routes (shown is the buccal cavity), then infects normal naïve B cells circulating through mucosal sites. Virus expresses type 3 latency, which drives B-cell proliferation and expands the infected memory pool. B-cell differentiation into the memory compartments occurs in germinal centers driven by type 2 latency proteins. Infected memory B cells exiting the germinal center down-regulate viral proteins and are invisible to the immune response. EBNA1 is expressed during homeostatic proliferation to maintain the latent viral episome. Virus replication is induced at mucosal sites, and virus is released into the saliva. PTLD indicates post transplantation lymphoproliferative disease; HD, Hodgkin disease; NPC, nasopharyngeal cancer, and BL, Burkitt lymphoma. Adopted from Heslop, (2009).

### **2.1.8.2 Reactivation of EBV from latency**

EBV hides in memory B cells and remains in a latent state to persist for lifelong period following primary infection. However, EBV periodically reactivates resulting in production of infectious virions. The precise mechanism that triggers EBV reactivation is unknown though it may serve as a means for virus to spread to uninfected B-cells. EBV protein Z (also known as Zta or ZEBRA) is important in the switch from latent to lytic cycle (Daud *et al.*, 2015).

### **2.1.9 Pathogenesis**

Infected B cells are one source of lymphomas, including Burkitt's and Hodgkin's lymphomas. The "civil war" which results between cytotoxic T lymphocyte (CTL) of the cell-mediated branch kill infected B lymphocyte is responsible for the symptoms and signs of IM (Bauman *et al.*, 2011).

The competence of cytotoxic T cells is in part related to age, thus the infection during childhood is usually asymptomatic because a child's cellular immune system is immature and cannot cause severe tissue damage. Whereas the infection if delayed until adolescence or later results in a more vigorous cellular immune response that produced the signs and symptoms of mononucleosis (Bauman *et al.*, 2011).

Extreme disease such as oral hairy leukoplakia (OHL) arises in individuals with a T cell deficiency, as occur in malnourished children, the elderly, Acquired Immunodeficiency Syndrome (AIDS) patients and transplant recipients. These individuals are more susceptible to EBV because infected cells are not removed by cytotoxic T lymphocytes and therefore remain a site of virus proliferation (Bauman *et al.*, 2011).

### **2.1.9 EBV and host immune response**

EBV infection induces a strong immune response comprising circulating antibodies against many virus-specific proteins, cell-mediated immune responses and production of lymphokines. The antibodies against viral membrane antigen confer lifelong immunity against the second attack of infectious mononucleosis (Parija, 2009).

Acute EBV infection is associated with activation and expansion of EBV-specific CTLs that gradually decline as viremia drops (Daud *et al.*, 2015), and cell-mediated immune functions return to preinfection levels, although memory T cells maintain the capacity to limit proliferation of EBV-infected B cells (Drew, 2010).

### **2.1.10 clinical syndrome**

Most primary infections in children are asymptomatic. In adolescents and young adults, about 50% of the classic syndrome associated with primary infection is IM (Butal, 2010).

Endemic BL is a monoclonal B cell Non-Hodgkin's lymphoma(NHL) characterized by a high proliferative index and is the most common childhood cancer in Equatorial Africa with a peak age of 6 years (Daud *et al.*, 2015).

The high incidence of NPC among the southern Chinese people suggests that genetic or environmental factors in addition to EBV may also be important in the pathogenesis of the disease (Drew, 2010). In AIDS patients, several distinct additional EBV-associated diseases may occur, including OHL (Drew, 2010), and hodgkin disease (Junker, 2005).

EBV also associated with gastric carcinoma (GC) (Junker, 2005), and X-linked lymphoproliferative syndrome (XLPS) which is a rare disease (Macswen and Crawford., 2003). Epidemiological data suggest that EBV

is associated with several autoimmune diseases, such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and multiple sclerosis (MS) (Lossius *et al.*, 2012).

### **2.1.11 Laboratory Diagnosis**

Isolation of EBV from clinical specimens is not practical, because this assay is laborious and time-consuming (6–8 weeks), requires specialized facilities, hence seldom used (Butal, 2010).

Nucleic acid hybridization is the most sensitive means of detecting EBV (Butal, 2010).

Molecular diagnosis by 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 IM. This EBV DNA level decline during convalescence. They are not demonstrated in latently infected individual (Parija, 2009).

EBNA 1 gene appears to be conserved between EBV strains without reported sequence differences between B95-8, JY and FF41 strains (Shibata *et al.*, 1991).

Laboratory analysis of EBV infectious mononucleosis is usually documented by the demonstration of atypical lymphocytes, and heterophile antibodies, or positive EBV-specific serologic findings (Drew, 2010).

Serologic procedures for detection of EBV-specific antibodies include enzyme linked immunosorbent assay (ELISA) tests and indirect immunofluorescence tests using EBV-positive lymphoid cells to detect specific antibody to viral antigens (VCA, EBNA) (Chakraborty, 2013).



### **2.1.12 TREATMENT**

The DNA polymerase enzyme of EBV has been shown to be sensitive to acyclovir, and acyclovir can decrease the amount of replication of EBV in tissue culture and in vivo (Drew, 2010). But does not affect the number of EBV-immortalized B cells (Butal, 2010). Laryngeal obstruction should be treated with corticosteroids (Drew, 2010).

### **2.1.13 Prevention and Immunization**

An EBV vaccine might confer enormous benefits in protecting against BL and nasopharyngeal carcinoma. An experimental subunit vaccine made from one of the viral envelope glycoproteins, gp340 has given promising results in Tamarind monkeys; nevertheless, the application of an EBV vaccine on a mass scale is still a long way off (Collier *et al.*, 2006).

Adoptive transfer of EBV-reactive T cells shows promise as a treatment for EBV-related lymphoproliferative disease (Butal, 2010).

## 2.2 Malaria

Malaria is a very old disease and prehistoric man is thought to have suffered from it. The term malaria is Roman in origin, although the disease was not known by its present name until the mid-eighteenth century. Before then it was referred to variously as ague, intermittent fever, swamp fever, Roman fever, and death fever. Malaria or "bad" or evil (mal) air (aire) was a name derived from the miasma theory of causation (Heggenhougen *et al.*, 2003).

Malaria typically is found in warmer regions of the world, in tropical and subtropical countries. Malaria parasites, which grow and develop inside the mosquito, need warmth to complete their growth before they are mature enough to be transmitted to humans (CDC, 2015).

Malaria parasite belong to the genus *plasmodium* . Four species of *Plasmodium* have long been recognized to infect humans in nature (*Plasodium falciparum*, *Plasodium malariae*, *Plasodium ovale* and *Plasodium vivax*). In addition there is one species (*P. knowlesi*) that naturally infects macaques which has recently been recognized to be a cause of zoonotic malaria in humans (CDC, 2015).

Usually, people get malaria by being bitten by an infective female *Anopheles* mosquito. Only *Anopheles* mosquitoes can transmit malaria and they must have been infected through a previous blood meal taken from an infected person. When a mosquito bites an infected person, a small amount of blood is taken in which contains microscopic malaria parasites. About 1 week later, when the mosquito takes its next blood meal, these parasites mix with the mosquito's saliva and are injected into the person being bitten (CDC, 2015).

Because the malaria parasite is found in red blood cells of an infected person, malaria can also be transmitted through blood transfusion, organ transplant, or the shared use of needles or syringes contaminated with blood. Malaria may also be transmitted from a mother to her unborn infant before or during delivery ("congenital" malaria) (CDC, 2015).

Microscopy remains the mainstay of malaria diagnosis in most large health clinics and hospitals but the quality of microscopy-based diagnosis is frequently inadequate (WHO, 2016).

In areas and situations where microscopical diagnosis of malaria is not available, the use of malaria rapid diagnostic tests (RDTs) can help to diagnose malaria promptly, improve the accuracy of malaria diagnosis, and avoid the unnecessary use of costly antimalarial drugs. Users of malaria RDTs, however, need to be aware of the limitations of RDTs, e.g. unlike microscopical diagnosis, most RDTs are less sensitive, are not able to estimate parasite density, cannot indicate when schizonts of *P. falciparum* are present in peripheral blood and cannot provide accurate species identification particularly for non-*P. falciparum* infections (Cheesbrough, 2009).

### **2.3 Role of malaria in EBV infection and Disease**

Acquisition of immunity to malaria is age dependent and occurs only after repeated infections. In addition, children living in areas where malaria transmission is holoendemic often have chronic, asymptomatic infections characterized by malaria parasites in the blood (Moormann *et al.*, 2005).

The mechanisms that may lead to viral reactivation during *P. falciparum* malaria are not well understood (Chêne *et al.*, 2007).

It is established that malaria parasites modulate and evade the host immune system. Indeed, these properties underlie the hypothesis that *P. falciparum* malaria suppresses immunity to EBV during coinfection (Chattopadhyay *et al.*, 20013). Two mutually compatible theories have been proposed to explain the relationship between EBV and *P. falciparum* malaria in the etiology of eBL (Chattopadhyay *et al.*, 20013).

The first suggests that malaria coinfection increases the number of latently infected B cells by inducing polyclonal B-cell expansion and consequent lytic EBV reactivation . In turn, the greater precursor frequency of EBV-infected B cells increases the likelihood of c-*myc* translocation, which is a hallmark of all BL tumors . The second theory argues that EBV-specific T-cell responses are selectively altered during malaria coinfection, either as a cause or consequence of enhanced EBV replication, leading to impaired viral control and/or immune surveillance (Chattopadhyay *et al.*, 20013).

The cystein-rich inter-domain region 1 $\alpha$  (CIDR1 $\alpha$ ) of the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) is one of the molecules involved in EBV reactivation during the course of malaria infection (Chêne *et al.*, 2007).

## 2.4 Background studies

Many studies support the hypothesis that repeated malaria infections in very young children modulate the persistence of EBV and increase the risk for the development of eBL (Moormann *et al*, 2005; Chêne *et al.*, 2007; Moormann *et al.*, 2007; Moormann *et al.*, 2011; Chattopadhyay *et al.*, 20013).

In a previous study, 73 Ghanaian children with and without acute malarial infection were included to study the occurrence and quantified cell-free EBVDNA in plasma (Rasti *et al*, 2005). Viral DNA was detected in plasma from Ghanaian children but was absent in plasma from Ghanaian adults and healthy Italian children. These findings provide evidence that viral reactivation is common among children living in malaria-endemic areas, and may contribute to the increased risk for endemic BL. The data also suggest that the epidemiology of EBV infection and persistence varies in different areas of the world.

Donati and his colleagues (2006) screened EBV DNA in Ugandan children by compared EBV DNA loads in plasma and saliva samples from 43 Ugandan children with acute malaria (age range, 2–15 years; mean age, 6.9 years) at the time of diagnosis and 14 days after antimalaria treatment, children without malaria (age range, 2–14 years; mean age, 6.3 years), and from 26 children with BL (age range, 3–10 years; mean age, 6.4 years). EBV DNA was detected, by real-time polymerase chain reaction. Antimalaria treatment led to clearance of plasma viral load in 85% of the cases but did not affect the levels in saliva. There was a significant difference in plasma EBV loads across the groups. The lowest levels were detected in samples from the malaria negative group, increased levels were detected in samples from the malaria positive group, and levels reached the highest values in samples from children with BL. The same

trend was evident in the frequency and levels of anti-BZLF1 antibodies, which is indicative of viral reactivation. In the malaria positive group, the positive plasma samples clustered around 7–9 years of age, the peak incidence of BL. The clearance of circulating EBV after antimalaria treatment suggests a direct relationship between active malaria infection and viral reactivation.

Other study compared the EBV loads in children living in 2 regions of Kenya with differing malaria transmission intensities: Kisumu District, where malaria transmission is holoendemic, and Nandi District, where malaria transmission is sporadic. For comparison, blood samples were also obtained from US adults, Kenyan adults, and patients with eBL. Extraction of DNA from blood and quantification by polymerase chain reaction give an EBV load estimate that reflects the number of EBV-infected B cells, and observed a significant linear trend in mean EBV load, with the lowest EBV load detected in US adults and increasing EBV loads detected in Kenyan adults, Nandi children, Kisumu children, and patients with eBL, respectively. In addition, EBV loads were significantly higher in Kisumu children 1–4 years of age than in Nandi children of the same age (Moormann *et al*, 2005).

## **CHAPTER THREE**

### **3- Materials and methods**

#### **3.1 Study design**

An analytical case-control study.

#### **3.2 Study area and Study duration**

This study was conducted at Al-jabel Military Medical Hospital and Jabel Aulia Hospital in the period from September to December 2015.

#### **3.3 Study population**

Study population included children with *P. falciparum* malaria infection aged between 1-15 years who attended to the hospitals at the time of the study. Diagnosis of the patients was based on clinical and laboratory procedures. Study population included also children with age matched those with *P. falciparum* malaria (control).

##### **3.3.1 Inclusion criteria**

Children at the age 1-15 years with or without *P. falciparum* malaria.

##### **3.3.2 Exclusion criteria**

Adults at the age  $\geq 16$  years.

#### **3.4 Sample size**

A total of 80 blood samples were collected in this study, included 50 from children with *P. falciparum* malaria infection and 30 children without *P. falciparum* malaria infection and apparently look healthy.

#### **3.5 Sampling technique**

Non-probability sampling technique.

### **3.6 Ethical clearance:**

Ethical clearance was obtained from local authorities in Al-jabel Military Medical Hospital and Jabel Aulia Hospital. Also permission of this study was taken from the Collage of Graduate Studies of Sudan University of Science and Technology.

### **3.7 Data collection**

#### **3.7.1 Method of data collection**

Data was collected from the patients and controls through direct interview (Appendix 1- questionnaire).

#### **3.7.2 Study variables**

Gender, Epstein–Barr virus DNA, malaria infection, density of parasite, age and Hemoglobin (Hb).

### **3.8 Site of experiment:**

Research laboratory of Collage of Medical Laboratory Science at Sudan University of Science and Technology.

### **3.9 Specimen collection:**

Blood specimens were collected in EDTA container and centrifuged at 3000 rpm for 10 minutes. Plasma was obtained and kept in cryovial tubes, then stored at -20 °C until tested.

### **3.10 Laboratory test:**

#### **3.10.1 Laboratory test for confirmation of malaria:**

Malaria infection was determined by conventional light microscopy on Giemsa stained thick and thin blood films.

For making thin blood film small drop of blood was added to the centre of the slide and a larger drop about 15 mm to the right, then spread the thin film using a smooth edged slide spreader. The spreader was placed in



front of the blood and was backwarded until it touched the blood and then moved forward by an angle 45 °C. Spread the large drop of blood to make the thick smear. The blood allowed to air-dry with the slide in a horizontal position, then finally by using a black lead pencil, the slide was labeled with date, patient's name and number (Cheesbrough, 2009).

Absolute alcohol was used to fix the thin blood film. Then the slide was placed in staining rack and covered by working Giemsa stain (10 ml of Giemsa stock solution to 90 ml of buffer solution) and allowed to stay for 10 minutes. The slide was washed to remove excess stain. After that was wiped with clean gauze, and then the slide was placed in slide drying rack to dry. The blood film was examined using microscope (Olympus, Japan) with oil immersion lens (100×) (Cheesbrough, 2009).

Malaria parasites were identified according to standard criteria. Trophozoites of *P. falciparum* small and large rings (ring form), were identified with double chromatin dot. And trophozoites of *P. vivax* were identified by their large and amoeboid fragmented cytoplasm and fine pigment (Cheesbrough, 2009).

The intensity of malaria parasitemia in positive blood smears were grouped into low(+), medium (++), and high (+++) on average number of parasites per microscopic field. The '+' indicates the relative parasite count as follows:

+ = 1-10 parasites per 100 thick- film field.

++ = 11-100 parasites per 100 thick- film field.

+++ = 1-10 parasites per one thick –film field.

++++ = more than 10 parasites per one thick-film field (Orji *et al.*, 2011).

### **3.10.2 EBV DNA extraction**

EBV-DNA was extracted from the plasma specimens by using commercial Kit (Vivantis, Malaysia), utilizing a specially-treated glass filter membrane technology for efficient recovery of highly pure DNA or RNA.

Firstly, 50µL of proteinase K was added to 200 µL of plasma, then 215 µL of Buffer VL (containing Carrier RNA) was added and mixed homogeneously by pulsed-vortexing then incubated at 65 C° for 10 minutes. 280 µL of ethanol was added and mixed thoroughly after that the solution was applied to a column and centrifuged at 5000×g for 1 min. The solution on collection tube was discarded and 500 µL of wash buffer1 was added and centrifuged at 5000×g for 1min. This step was repeated twice for wash buffer 2, but in second time was centrifuged at maximum speed for 3min. The column was transferred into a clean microcentrifuge tube, 35µl of elution buffer provided with the kit was added then left for 2 min and centrifuged at 5000×g for 1 min. Finally the eluted DNA was stored at - 20 C° until further analysis.

### **3.10.3 Polymerase chain reaction for EBV**

The amplification and detection of EBV DNA was carried out by conventional PCR method in thermal cycler PCR machine (Techne, Japan) using primers set that amplify an 80-base pair region of EBNA1 gene. Sequences of EBV primers were obtained from published data (Mizobuchi *et al.*, 1997) and were as follows:

SL 1 5-GGACCTCAAAGAAGAGGGGG-3

SL 3 5-GCTCCTGGTCTTCCGCCTCC-3

The PCR reaction mixture contained in addition to 5 µl of the purified DNA, 2 µl primers, 13µl water for injection, and 5 µl i-Taq ready to use

master mix (iNtRON Biotechnology- Korea) in a 25 µl total reaction volume.

The samples were first denatured at 94 °C for 10 min, then thermocycled for 35 cycles (45 sec at 95 °C , 45 sec at 60 °C , 60 sec at 72 °C), and final extension was done at 72 °C for 10 min (Mizobuchi *et al.*, 1997).

#### **3.10.4 Gel electrophoresis**

3.5% Agarose gel was prepared by adding 3.5 g of agarose in 100 ml of 1X TBE buffer (Tris Boric acid EDTA buffer), then stained with 4 µl ethidium bromide. 10 µl of PCR products were loaded into each well of the prepared gel (Mizobuchi *et al.*, 1997). 50 bp molecular weight marker was loaded on either ends of the gel to score amplified bands. The TBE buffer was ensured not contain precipitates. Gel electrophoresis was run at 100 volts for 45 minutes. The gel was scanned by using gel documentation system (Syngen, UK).

#### **3.10.5 Interpretation of PCR results**

A positive sample for EBV produced a band of 80 bp for EBNA1 gene (Mizobuchi *et al.*, 1997).

#### **3.11 Data analysis:**

Data analysis was done using statistical package of social science program (SPSS version 11.5).

## CHAPTER FOUR

### 4-Result

In this study 80 children, 47 (58.8%) males and 33 (41.2%) females, within the age range of 1-15 years were examined. The study population was divided into study group which composed of 50 children infected with *P. falciparum* malaria (mean age  $7 \pm 4$  years), of them 31(62.0%) were males and 19 (38.0%) were females, and control group which composed of 30 children of age matched those with malaria infection. The mean age of this group was  $7.6 \pm 4$  years, of them 16 (53.3%) were males and 14 (46.7%) were females.

Mean of Hb levels was  $9.8 \text{ g/dl} \pm 2.4$  for the study group and  $10.4 \text{ g/dl} \pm 2$  for the control group.

The confirmatory microscopic examination of blood film for malaria diagnosis revealed that *P. falciparum* was the predominant species [49 (98%)] of malaria in the area of study. Only one child was suffering from malaria caused by *P.vivax*. The intensity of malaria parasitemia in positive blood films were grouped into low(+) and medium (++); accounting 96% and 4%, respectively.

Among the study group, there was no detection of EBV, while control group showed amplification of DNA of about 80 bp corresponding to the product amplified by the specific primers, a common region of EBNA1 gene. Based on their size, two specimens were considered positive for EBV- DNA (**Fig. 4.1**).

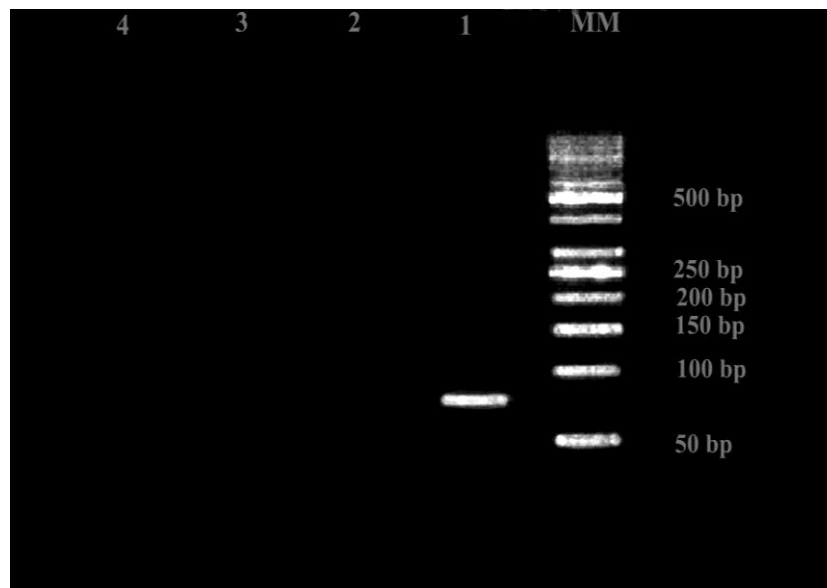
Frequency and percentage of EBV- DNA (EBNA1) among *P. falciparum* infected children (study group) and non-malaria infected children (control group) was shown in **Table 4.1**. Compared to 0% positivity for *P.*

*falciparum* malaria infected children, EBV- DNA was detected in 6.7% of the non-*P. falciparum* malaria infected children.

The positivity for EBV-DNA according to age group of study population was shown in **Table 4.2**. The two children of control group were in the younger age group, 1-5 years of age. The difference between positivity of EBV-DNA and the age group of the control group was not significant (*P. value* = 0.2).

The frequency of EBV-DNA according to gender among the control group showed no significant difference (*P. value* = 0.922) (**Table 4.3**).

In addition there was no differences between the EBV-DNA and parasitemia, and Hb levels (*P. value* = 0.392). The two individuals with positive EBV-DNA were from the high Hb level group, 11-15 g/dl (**Table 4.4**).



**Fig. 4.1: Gel electrophoresis for amplification of EBNA1 gene.** Primers (SL1 and SL3) targeting a conserved region in EBNA1 gene were used. The amplified product corresponding to 80 bp was detected in 3.5% agarose after staining with ethidium bromide and visualized in UV light. Lane 1 showing amplification of DNA of about 80bp, representing one of the two specimens of control group. Lanes 2 and 3, two specimens of *P. falciparum* infected patients showing negative results for EBNA1 gene of EBV; Lane 4, negative control. MM, 50 bp Molecular marker DNA.

**Table 4.1: Frequency and percentage of EBV DNA among *P. falciparum* malaria infected and control group**

Study population	EBV- DNA	
	Positive	Negative
<i>P. falciparum</i> infected children (n=50)	0 (0.0%)	50(100%)
Control group (n=30)	2 (6.7%)	28(93.3)
Total (n=80)	2 (2.5%)	78(97.5%)

Values are number (percentage). EBV, Epstein-Barr virus; DNA, deoxyribonucleic acid.

**Table 4.2: Frequency of EBV-DNA among *P. falciparum* malaria infected children and control group according to the age group of Study population**

Age group	<i>P. falciparum</i> infected children		Control group		Total
	EBV-DNA		EBV-DNA		
	Positive	Negative	Positive	Negative	
1-5 years	0 (0%)	21 (42%)	2 (6.7%)	10(33.3%)	33 (41.%)
6-10years	0 (0%)	18 (36%)	0 (0%)	9 (30.0%)	27 (33.%)
11-15years	0 (0%)	11 (22%)	0 (0%)	9 (30.0%)	20 (25%)
Total	0 (0%)	50 (100%)	2 (6.7%)	28 (93.3%)	80(100%)

Values are number (percentage). EBV, Epstein-Barr virus; DNA, deoxyribonucleic acid.



**Table 4.3 Frequency of EBV-DNA among the *P. falciparum* malaria infected children and control group according to gender**

Study population	Gender	EBV-DNA	
		Positive	Negative
<i>P. falciparum</i> infected children (n=50)	Males	0 (0.0%)	31(62.0%)
	Females	0 (0.0%)	19(38.0%)
Non-malaria infected children (n=30)	Males	1(3.3%)	15 (50%)
	Females	1(3.3%)	13 (43.3%)
Total (n=80)		2 (2.5%)	78(97.5%)

Values are number (percentage). EBV, Epstein-Barr virus; DNA, deoxyribonucleic acid.

**Table 4.4 Frequency and percentage of EBV-DNA among control group according to Hb levels**

<b>Hb levels g/dl</b>	<b>EBV- DNA</b>		<b>Total</b>
	<b>positive</b>	<b>Negative</b>	
1-5	0 (0%)	1 (3.3%)	1 (3.3%)
6-10	0 (0%)	13 (43.3%)	13 (43.3%)
11-15	2 (6.7%)	14 (46.7%)	16 (53.3%)
Total	2 (6.7%)	28 (93.3%)	30 (100%)

Values are number (percentage). EBV, Epstein-Barr virus; DNA, deoxyribonucleic acid, Hb; heamoglobin.

## CHAPTER FIVE

### 5-Disscution

#### 5.1 Disscution

EBV infects more than 95% of the human population. African children are infected early in life, and nearly all have seroconversion by 3 years of age (Donati *et al*, 2006). Cell-free EBV-DNA is not frequently detected in plasma from healthy seropositive donors but commonly found in plasma from patients with infectious mononucleosis or with EBV-associated malignancies. After primary infection, the presence of EBV-DNA in plasma or serum has been interpreted as diagnostic of active EBV replication (Rasti *et al*, 2005).

In this study, EBV-DNA was not detected in children infected with *P.falciparum*. In contrast, in the control group, positive results were obtained in 2 specimens, representing 6.7% positivity. This finding discord with those previously reported among children with malaria infection in Uganda (Donati *et al*, 2006), and Ghana (Rasti *et al*, 2005). In these studies EBV- DNA greaterly detected in malaria positive group (31%), (47%) than malaria negative group (18%), (34%), respectively. These studies support the hypothesis that the viral reactivation is common among children living in malaria-endemic areas, Donati and his collages suggested that the epidemiology of EBV infection and persistence varies in different areas of the world.

This variation in the result may be related to several factors such as variation in the sample size, study design, study area, protocol of EBV-DNA detection, or primers and target gene. In Donati *et al* (2006) study primers targeting EBV LMP1 gene were used, suggesting better diagnostic value of these gene. In addition, other factors also may play a

role such as study population, prevalence of EBV and its strains, the factor of climate and temperature difference, the circulation and the pathological impact of both pathogen might show variation.

In this study, collected data did not involve information if those children had received antimalaria within 72 hr before the examination. It have been reported that the antimalaria treatment leads to clearance of plasma EBV DNA viral load in 85% of the cases, therefore a direct relationship between active malaria infection and viral reactivation has been suggested (Donati *et al*, 2006). In addition, there is lack of information on prevalence of EBV infection among healthy Sudanese children to identify the age of primary infection by EBV. Available data is related to children with cancer (Ahmed *et al*, 2012; Osman *et al.*, 2014 ; Bilal, 2015 ).

The presence of EBV-DNA in plasma specimens of non-malaria infected group although not infected with malaria at the time of the study, may be explained as have been infected with malaria in the past. Moreover, children living in these areas are often exposed to many other infectious/parasitic and non parasitic diseases and have a relatively poor nutritional status that could indirectly contribute to impaired control of EBV persistence (Rasti *et al*, 2005).

In this study the results for presence of EBV-DNA in samples from the younger age group (1-5 years of age) were similar to that reported earlier. Moormann *et al* (2005) in Kenya and Njie *et al* (2009) in Gambia, have reported that the rate of EBV-DNA detection tended to be high in younger age group; 1-4 years, 3-5, respectively. These results it was differ from those reported in Uganda by Donati *et al* (2006). These authors have shown that the common age group was 7-9 years old.

There was no differences between the EBV- DNA and factors such as patient's age, gender, parasitemia, and Hb level and this findings were harmonized with previous studies (Rasti *et al*, 2005 and Donati *et al*, 2006).

Other study conducted in Sudan on EBV associated pediatric leukemia EBV LMP1 messenger RNA was detected by PCR in 29 (36.3%) of the 80 cases and in none of the 20 healthy controls, and possibly suggested the role of primary EBV infection in those children (Ahmed *et al*,2012).

In the first report on the prevalence of EBV infection among children with cervical lymphadenopathy in Sudan and the available literature on prevalence of EBV infection in children, the EBV infection was diagnosed in 15.9% of children with cervical adenopathy. Their results showed also that the younger children (age  $\leq 9$  years) are less likely to be sero-positive for EBV- IgM (Bilal, 2015).

EBV genome was detected in Sudanese patients (age range 10 - 80 years) with nasopharyngeal carcinoma in 77.6% and 84.5% of the tumors by EBNA-1 and LMP-1 primers, respectively (Adam *et al.*, 2011).

## **Conclusion**

Differently from what have been reported earlier, the results of the current study may indicate low or absence of EBV infection in *P. falciparum* malaria infected children or low prevalence of EBV infection among child in the study area. However, a major limitation of this study is the small sample size.

## **Recommendations**

1. Further studies are needed to assess the seroprevalence of EBV infection among the children in Sudan.
2. A larger scale longitudinal survey will provide further information on this topic.

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# Appendices

## Appendix-1: Questionnaire

### Sudan University of Science and Technology

#### Detection of Epstein–Barr Virus DNA in Children with or without *Plasmodium falciparum* Malaria Infection in Jabel Aulia - 2015

##### Part one:

No. : .....

Name: .....

Gender:.....

Age: .....

Residence: .....

Investigation results:

BFFM:.....

HB:.....g/dl

##### Part two:

##### Laboratory section:

1) Confermetory test for malaria:

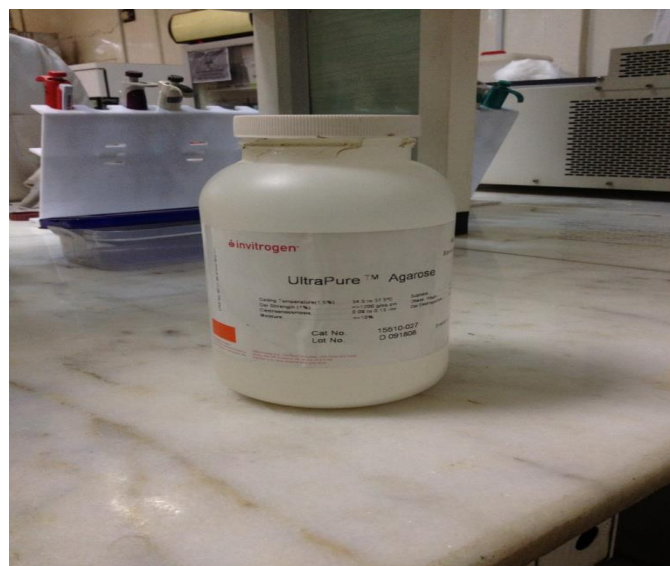
BFFM:.....

2) Results of Polymerase chain reaction for EBV DNA:

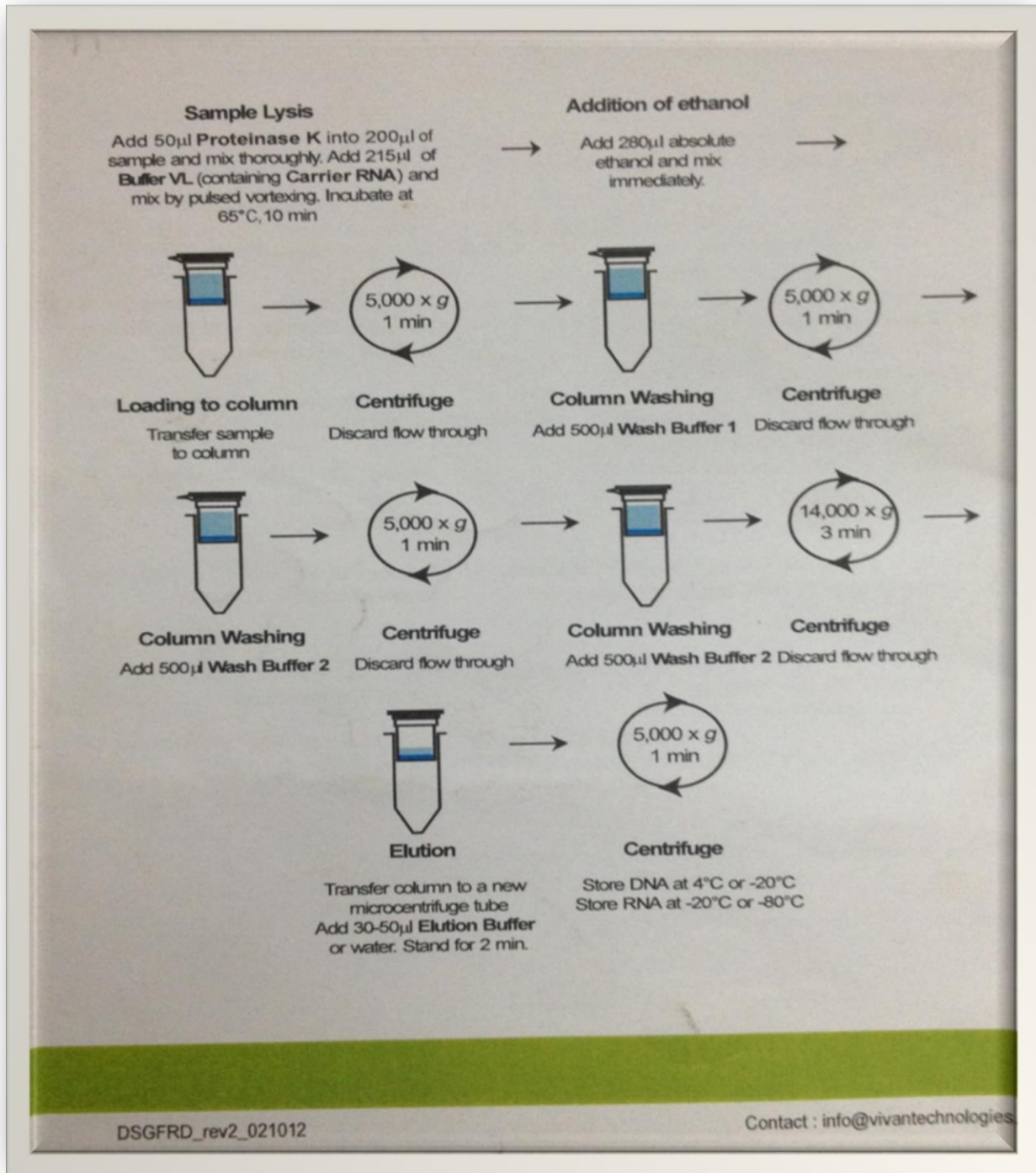
.....



**Appendix (2): Thermocycler machin (Techne, Japan).**



**Appendix (3): Agarose powder (UltraPure-  
invitrogene).**

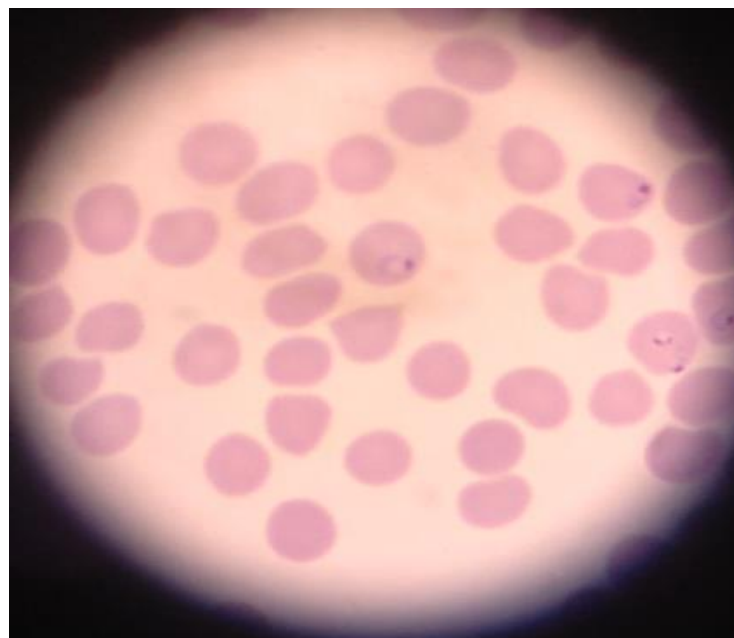


**Appendix (4): Protocol of DNA extraction (Vivantis, Malaysia).**





**Appendix (5): GF-1 Viral Nucleic Acid Extraction Kit (Vivantis-Malaysia).**



**Appendix (6): Trophozoites of *P. falciparum*.** The picture shows small and large rings stage (ring form) of *P. falciparum*. Adopted from my mobile.