# EPSTEIN-BARR VIRUS INFECTIONS IN PATIENTS WITH ACUTE FEBRILE ILLNESS IN KENYA

**By**

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**SCHOOL OF BIOLOGICAL SCIENCES**

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## DECLARATION

I declare that what has been presented here is my own original work and has not been presented in any other institution or university for the award of degree.

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We (the supervisors) confirm that the research findings in this thesis were carried out by the said candidate under our supervision.

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# DEDICATION

*To my loving late Mum and Dad,*

*Brother Nicholas, and*

*Sisters Bertha, Auma and Elizabeth*

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# ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| AFI | Acute Febrile Illness |
| AIDS | Acquired Immunodeficiency Syndrome |
| CDC | Centers for Disease Control |
| $C_T$ | Threshold Cycle |
| CTL | Cytotoxic T Lymphocyte |
| DOMC | Division of Malaria Control |
| DNA | De-oxyribo Nucleic Acid |
| EBER | Epstein-Barr Virus Encoded RNA |
| eBL | Endemic Burkitt Lymphoma |
| EBNA | EBV Nuclear Antigen |
| EBV | Epstein-Barr Virus |
| HHV-4 | Human Herpes Virus 4 |
| HIV | Human Immuno-deficiency Virus |
| IM | Infectious Mononucleosis |
| KEMRI | Kenya Medical Research Institute |
| LMPs | Latent Membrane Proteins |
| NASCOP | National AIDS and STI Control Programme |
| PTLDs | Post-transplant Lymphoproliferative Disorders |
| RNAs | Re-oxyribo Nucleic Acid |
| qRT PCR | Quantitative Real Time Polymerase Chain Reaction |
| VCA | Viral Capsid Antigen |
| WHO | World Health Organization |
| WRP | Walter Reed Project |
| WRAIR | Walter Reed Army Institute of Research |

# ABSTRACT

Epstein Barr Virus (EBV) has emerged as an important etiologic agent of infectious mononucleosis and still-growing number of lymphoproliferative disorders. It is also a potential cause of acute febrile illness (AFI). At present, the viral-prevalence of EBV and its association with acute febrile illness have not been adequately studied in Africa. This study sought to catalogue the prevalence and viral loads of EBV in patients with acute febrile illness in Kenya. A voluntary hospital based cross-sectional study of EBV infection was conducted between September 2008 and April 2013 on 2107 patients presenting with AFI at eight out-patient clinics across Kenya. EBV was detected in whole blood by a TaqMan-based real-time PCR for the BALF5 region of EBV genome. The primers and probes used were obtained from commercial sources (Applied Biosystems, Foster City, CA, USA), and the positive control was purchased from Vircell Microbiologists, Granada, Spain. Prevalence rates and viral load level were determined and examined according to demographic characteristics, region of residence, malaria endemicity and malaria infection. EBV was detected in 29.1% (614/2,107) of the recruited AFI patients. Their viral load ranged from 52 to 7.19 $x10^6$ copies/mL (mean 35,699 copies/mL; median 4,699 copies/mL). Viral prevalence was highest in patients aged below 5 years but reduced with increase in age. Of the 614 positive patients, 443 (72.1%) had viral load above the 2,000 copies/mL cutoff that is considered clinically important. Again the <5 year olds constituted the majority in this group ($p<0.05$). Regionally, the Lake Victoria and the coastal regions were found to have higher prevalence rates compared to the highland of Kisii and semi-arid areas ($p= 0.1636$). Of the following clinical features that were commonly associated with AFI, chills, cough, sore throat, headache, runny nose, abdominal pain, joint aches and muscle pain, EBV viral load significantly associated with runny nose ($p=0.0006$). Regarding malaria endemicity,

incidences of EBV infection were higher (35.7%) in regions considered holoendemic for malaria (L. Victoria basin and coast) compared to (22.9%) for hypoendemic regions (highland of Kisii and semi-arid areas) ($p<0.05$). Finally, comparison of viral loads between AFI patients co-infected with EBV and malaria and those with EBV infections alone revealed higher viral loads in the co-infected group (5,329 copies/mL compared to 3,655 copies/mL, $p= 0.004$), and this difference was much more pronounced in the 5-9 year olds. The study demonstrates that EBV is a common infection among patients with AFI; hence the need to create more awareness among healthcare workers that EBV can be an important cause of AFI. The age, malaria endemicity, and infection with malaria influence EBV prevalence and viral load levels. Like other AFI, clinical features are not helpful in arriving at EBV diagnosis. The findings further suggest the need to investigate whether increased EBV viraemia in EBV-malaria co-infections among the 5-9 year olds is a precursor to the development of Burkitt lymphoma.

Key words: Epstein-Barr virus, acute febrile illness, viral load and Malaria

# CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

## 1.1. Introduction

Acute febrile illnesses (AFI) are defined as non-specific illnesses presenting with fever $\geq 38^0$C without a readily diagnosable source after routine clinical evaluation (Cunha, 2007; Chow & Robinson, 2011). They are the most common causes of outpatient attendance and mortality, especially among children in Africa (Sykes *et al*., 2011). The underlying pathogens are largely unknown in Africa partly due to limited diagnostic facilities for infectious diseases (WHO, 2011). Because of the high prevalence and mortality associated with malaria, most AFIs are assumed to be malaria and managed as such even in absence of the parasites. If the patient doesn't get better, it is assumed that the fever was caused by a resistant malaria strain and another anti-malarial treatment is prescribed (Bojang *et al.,* 2000). However, with the ongoing vigorous malaria control and prevention campaign, it has now become clear that clinical presentation alone is not diagnostic of malaria. This has generated interest to explore for other infectious agents of AFI so as to plan for disease-targeted management. Recent data indicate that Epstein Barr virus (EBV), *Mycobacterium tuberculosus*, *Salmonella*, *Brucella*, *Rickettsia*, *Leptospira*, Cytomegalovirus and Human Immuno-deficiency Virus (HIV) can be important causes of AFI (Dilek *et al.,* 2011).

Epstein Barr virus, also known as Human herpes virus 4, is a very common viral infection in the world and is an emerging cause of AFI globally (ref). Serological studies have shown that over 90% of the world's adult human population has once been infected with EBV (Rickinson, 2002; Rezk & Weiss, 2007). Most infected individuals remain healthy for the rest of their lives but a few may develop symptomatic infection commonly characterized by fever (Thompson & Kurzrock, 2004). Other patients may present with lymphadenopathy, pharyngitis, abdominal

discomfort, palatal petechaie, rash, headache, fatigue, malaise, ulceration splenomegaly, hepatomegaly, jaundice, and periorbital oedema. Some may develop complications such as autoimmune hemolytic anemia, splenic rupture, hemophagocytic lymphohistiocytosis, and neurological complications which can be life- threatening (Hess, 2004). The disease generally lasts for about 1 to 4 weeks (Thompson & Kurzrock, 2004). The virus has increasingly become important in immunosuppressed individuals, either from increased cases of HIV or due to increasing cases of transplant and cancer chemotherapy. Primary infection and possible reactivation in the body may not only lead to occurrence of severe fever but can give rise to a wide variety of benign and neoplastic diseases ranging from Hodgkin's lymphoma to Burkitt lymphoma, post-transplant lymphoproliferative disorders (PTLDs), and oral hairy leukoplakia in AIDS patients (Gulley, 2001; Rezk & Weiss, 2007).

Making correct diagnosis for a cause of febrile illness remains a public health challenge in many healthcare facilities (WHO, 2011). For EBV induced febrile cases, reliable diagnosis should include detecting and quantifying DNA load in clinical samples. Although the correlation between EBV burden and disease status is incompletely understood, recent clinical studies suggests that whole blood EBV loads can be essential in measuring pathogenesis and risk of developing EBV-associated disorders (Stevens *et al*., 2001; Stevens *et al.*, 2005). There are several methods of detecting and quantifying EBV viral load. These include dot blotting, Southern blotting, *in situ* hybridization, conventional PCR, and real-time PCR methods (Lay *et al.*, 2010). The advent of real time qPCR has revolutionized diagnosis and monitoring EBV-associated diseases. It is generally preferred for its sensitivity, specificity, speed and ease of handling (Leung *et al.,* 2002; Luderer *et al.,* 2005; Lay *et al*., 2010). It has become an important tool for early diagnosis of infections as well as a technique to monitor response to treatment. By

use of quantitative real time PCR, Stevens *et al.* (2001 & 2005) have explained that a cutoff value of 2000 EBV DNA copies /ml of blood clearly demarcates patients with EBV-associated disorders from healthy EBV carriers. A study by Aalto *et al* (2007) has suggested that individuals with EBV DNA levels ≥ 50 000 copies/mL should be considered to have life-threatening EBV infection. A viral load of up to 120,000 copies/mL may indicate a case of non-Hodgkin's lymphoma in Aids patients while elevated viral loads of up to 4,592,000 copies/mL of blood may indicate cases of Burkitt lymphoma (Stevens *et al.*, 1999; Meerbach *et al.*, 2001).

In Kenya, EBV is well known as a cofactor in the development of endemic Burkitt lymphoma (eBL). This is the most common pediatric cancer accounting for 70% of cancer related childhood mortality in equatorial Africa (Moormann *et al.*, 2011). Although it is widely known due to its association with eBL, there is little data on its viral-prevalence and association with acute febrile illness. Using quantitative Real Time PCR technique, this study sought to investigate EBV infection in febrile patients attending hospitals in different parts of the country so as to determine its role in the occurrence of acute febrile illness.

## 1.2. Literature review

### 1.2.1. Epidemiology of EBV

Epstein Barr Virus is extremely ubiquitous and is believed to be harbored by more than 90% of the world adult human population (Rickinson, 2002; Rezk & Weiss, 2007). It is a tumorigenic human herpes virus that mainly infects B-cells and epithelial cells (Hassan *et al*., 2006). It is known to be the causative agent of infectious mononucleosis (IM), an acute but a self-limiting clinical syndrome that mainly afflicts children and adolescents in the developed countries (Gulley, 2001; Williams & Crawford, 2006; Rezk & Weiss, 2007; Lay *et al.*, 2010). EBV is also

implicated in the etio-pathogenesis of still-growing number of a wide variety of benign and neoplastic diseases. This include B-cell lymphoproliferative disorders such as Hodgkin's lymphoma, Burkitt lymphoma, and post-transplant lymphoproliferative disorders (PTLDs); T-cell lymphoproliferative disorders such as peripheral T-cell lymphomas, angioimmunoblastic T-cell lymphoma, and extranodal nasal type natural killer/T-cell lymphoma (Carbone *et al.,* 2008). It is also involved in the occurrence of epithelial tumors ranging from nasopharyngeal carcinoma to Non-Hodgkin's lymphoma to gastric carcinoma and oral hairy leukoplakia (Gulley, 2001; Rezk & Weiss, 2007).

In the more industrialized societies where substantial proportions of the population enjoy higher socioeconomic status, primary infection mainly occurs during the adolescence or early adulthood (Haahr, *et al*., 2004). In these developed societies, about 50% of the young adults develop the febrile-like syndrome commonly referred to as infectious mononucleosis or glandular fever (Tarbouriech *et al.,* 2006). In the underdeveloped regions such as Africa, Southeast Asia and Latin America, primary infection is more frequently acquired during childhood and is often thought to be asymptomatic (Figueira-Silva & Pereira, 2004).

Variation in the distribution and prevalence of EBV infection in the world has been shown to occur with the main contributing factors being socio-economic, geographical and hygienic conditions (Thompson & Kurzrock, 2004). In the United States, a study conducted in the military academy at West Point showed that the rate of EBV prevalence varied in relation to the geographic area within the United States from which the cadet originated (Hallee *et al.,* 1974). Those from west, north central, and New England states had an EBV prevalence of about 50% while those from the south central and east central states had an EBV prevalence that exceeded 75%. In addition, the same authors found out that the prevalence varied with the economic status,

with rates averaging 77% in cadets from families with incomes under $6000, in contrast to rates of 59% in cadets from families with incomes above $30 000. These data sets were reinforced by other studies that demonstrated a strong correlation of high EBV prevalence with low family income and education level (Figueira-Silva & Pereira, 2004). Their results also showed that the prevalence increases with the age, hence indicating that almost all people in the world become infected during their lifetime

### 1.2.2. Taxonomy, structure and biology of EBV

#### 1.2.2.1. Taxonomy

Epstein Barr virus or human herpes virus (HHV)-4 is a member of herpesvirus family, belonging to the *genus Lymphocryptovirus.* Named after the discoverers, Michael Anthony Epstein and Yvonne Barr, EBV was first identified using electron microscopy in a small percentage of cells cultured from Burkitt lymphoma in 1964 (Epstein *et al.*, 1964; Cohen, 2000). It is a persistent, latent and contagious agent that has co-evolved with humans for over millions of years and as a result, it has become one of the most well adapted herpes viruses in the human population.

Two strains of EBV, that is EBV-1 and EBV-2, infect humans. The strains differ in the sequences of EBV nuclear antigen (EBNA) gene (Sample *et al.*, 1990). EBV-1 infection is more prevalent in North-America and Europe while EBV-2 infection is more prevalent in Africa (Yao *et al.*, 1998). HIV patients may be co-infected with more than one strain of EBV-1 or EBV-2, with both EBV-1 and EBV-2, or with intertypic recombinants. In the United States and Europe, a much higher frequency of EBV-2 has been reported in HIV positive individuals (Yao *et al.*, 1998).

#### 1.2.2.2. Viral structure

EBV is an enveloped, icosahedral, double stranded DNA virus with a diameter of 120 to 220 nm. Its DNA molecule is approximately 186 kilo base pairs in length, and consists of unique long domains, internal repeats, and terminal repeat domains (Hess, 2004). The internal repeat sequences serve to divide the genome into short and long unique sequence domains that have most of the coding capacity (Kieff *et al*., 1982) (Fig.1). The genome encodes 86 proteins (Tarbouriech *et al*., 2006), majority of which are essential for regulating the expression of viral genes, replicating viral DNA, forming structural components of the virion, and modulating the host immune response. EBV DNA is surrounded by an icosahedral nucleocapsid that consists of 162 capsomers. This nucleocapsid is covered by a protein tegument which is, in turn, enclosed by the viral envelop that consists of multiple viral glycoproteins (Liebowitz & Kieff, 1993).

#### 1.2.2.3. Life cycle

Figure 2 shows the schematic presentation of EBV life cycle in the human host. The virus primarily infects squamous epithelial cells in the oropharynx and B lymphocytes (Gulley, 2001). Infection begins with attack of epithelial cells of the oropharynx following the contact of EBV virus with oral secretions. The virus initiates lytic infection within the epithelial cells resulting into mass production of virus which consequently causes the cells to burst open (Cohen, 2000). How the virus accesses these epithelial cells is less well understood, because such cells are largely resistant to infection with cell-free virus *in vitro* (Shannon-Lowe *et al.,* 2006). B lymphocytes become infected when they come into contact with infected epithelial cells. While within the B cells, the virus disseminates to other body parts via the bloodstream. Survival of the virus inside the B cells results into latent infection and immortalization of the cells. When the B lymphocytes return to the oropharynx, they occasionally undergo plasma-cell differentiation,

which triggers viral replication and  eventually results into release of virus in saliva for spreading to other hosts or may infect more B lymhpcytes (Cohen, 2000; Young & Rickinson, 2004).

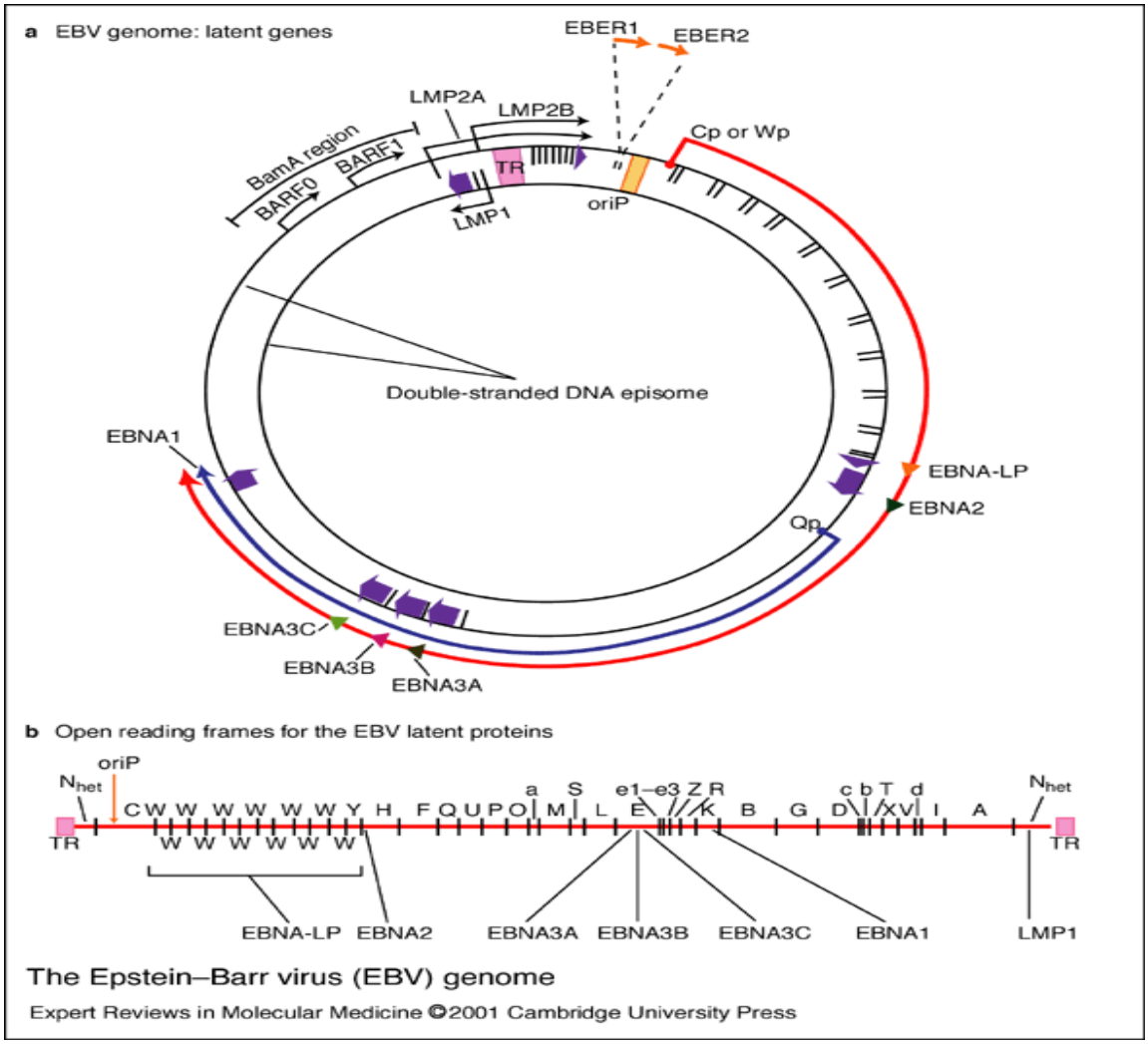


**Figure 1.** The general structure of Epstein Barr virus genome (Young & Rickinson, 2004)

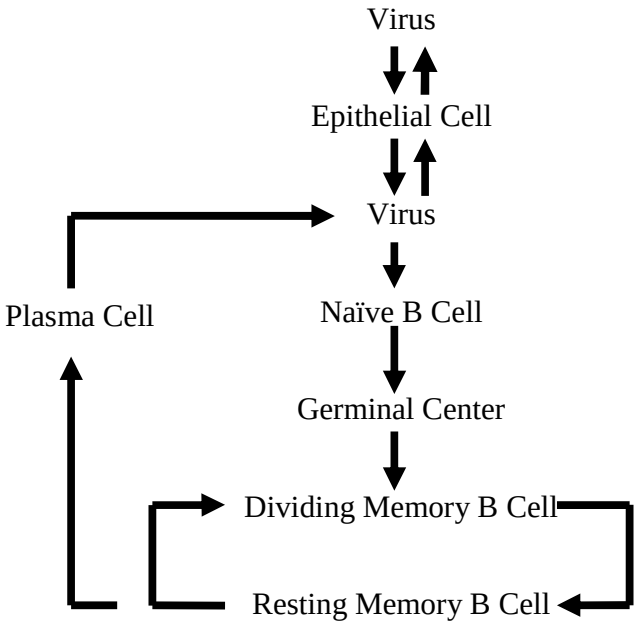


**Figure 2.** Schematic presentation of EBV life cycle (Thorley-Lawson, 2005)

#### 1.2.2.4. Latent infection and transformation

EBV latent infection of B lymphocytes is crucial for virus persistence, subsequent replication in the epithelial cells, and the release of infectious virus into saliva. According to Babcock *et al.* (1998), the resting memory B cells are the site of persistence of EBV within the body. Their study also indicated that in a normal adult, 1 to 50 cells per million in the circulation are infected with EBV, and that the number of latently infected cells within a person remains stable over years. During this type of infection, only a few of the nearly 100 EBV genes are expressed. These latent genes include six EBNAs, two latent membrane proteins (LMPs), two EBV encoded RNAs (EBERs), and transcripts from the *BamHI A* region of the genome. This latent infection is advantageous to the virus in that it limits viral gene expression, thus reducing the number of proteins that permit the recognition of infected cells by cytotoxic T cells.

Following a successful latent infection, EBV virus efficiently induces B cells to undergo transformation to permanent lymphoblastoid cell lines in a multistep process. The process involves induction of mature B cells to enter into the cell cycle (going from a Go, or resting state, to G1). The EBV-infected cells then begin to proliferate in a manner that depends on high cell density and on the autocrine production of B cell growth-promoting cytokines. The EBV-infected B-cells continues to proliferate for over a period of time, and evolve into more rapidly growing cells that are less dependent on autocrine growth mechanisms (Kieff, 1994).

#### 1.2.2.5. Patterns of latency

Four different patterns of latency have been associated with EBV infection. In latency 1, only EBNA-1 protein is expressed in addition to the EBERs and BamHI A transcripts. This pattern of gene expression is typically observed in tissues of patients with Burkitt lymphoma (Sbih-Lammali *et al.,* 1996). Latency 2 is characterized by the expression of EBNA-1, LMP-1 and LMP-2. This pattern is seen in tissues of patients with Hodgkin's disease, peripheral T-cells, and nasopharyngeal carcinoma (Cesarman & Mesri, 1999). Latency 3 is characterized by the expression of all the EBV-associated latent proteins together with the EBERs and BamHI A RNAs, and it is usually seen in immunocompromised individuals with EBV associated lymphoproliferative disease and in lymphoblastoid cell lines (Niedobitek *et al.,* 1994). The fourth latency pattern comprises expression of LMP-2 transcripts and, in some cases, EBNA-1(Tierney *et al.,* 1994).

#### 1.2.2.6. Immune Responses to EBV

Infection with EBV stimulates both cellular and humoral immune responses with the former being the most important for the control of this infection (Cohen, 2000). During primary infection, natural killer cells, helper T cells, and Cytotoxic T cells eliminate large numbers of

EBV-infected B-cells by responding to about 100 different antigens (Rickinson & Moss, 1997). This cell mediated response is probably responsible for the symptoms of fever, lymphadenopathy, and splenomegaly experienced during the acute EBV infection. Immunity in the latent phase mainly depends on HLA- restricted cytotoxic T lymphocytes (CTLs) which act by lysing the expressed EBNAs and LMP proteins. Many of CTL responses are however directed to EBNA-3 proteins, thus, in the instances of BL and nasopharyngeal carcinoma where only EBNA-1 appears to be produced, the virus evades destruction (Uchida *et al.*, 1999). Humoral response during this infection is evident by the elevated antibody titers to Viral Capsid Antigen (VCA) and Early Antigen (EA) observed in most infected persons. Existence of autoantibodies has also been observed. According to Sairenji (1997), proliferation of specific B-cell clones during reactivation leads to the production of autoantibodies for the glycine-alanine repeat region of EBNA-1.

### 1.2.3. EBV transmission

EBV transmission occurs primarily through saliva (Haahr *et al.*, 2004). In low socioeconomic societies, infection is acquired in early childhood. Crowding and the practice of pre-chewing food for infants is said to be a contributing factor (de The' et al., 1975). In the industrialized societies, infection is frequently spread during the adolescence stage due to intimate oral exposure (Hjalgrim *et al*., 2007). Primary infection in the adolescence stage develops infectious mononucleosis in approximately half of the cases (Tarbouriech *et al*., 2006). EBV has also been found in male and female genital secretions suggesting that it can be transmitted sexually (Adjei *et al*., 2008). Blood transfusion, bone marrow transplantation, and solid organ transplantation are believed to be other means of transmission (Junker, 2005; Adjei, et al., 2008). Mother to unborn

transmission via the placenta and breast feeding has also been reported in rare circumstances although they are now considered non-significant modes of transmission (Haahr, *et al*., 2004).

### 1.2.4. Diagnosis of EBV infection

The diagnostic approaches for EBV infection vary depending on the immune status of the patients. They include physical examination, serological tests and polymerase chain reaction based methods. Physical examination involves identifying an array of clinical characteristics with the most predominant ones being fever, pharyngitis, and lymphadenopathy. Other clinical symptoms such as sore throat, headache, fatigue, malaise, ulceration, hepatomegaly, jaundice, periorbital oedema abdominal discomfort, palatal petechaie and splenomegally are also associated with acute EBV infection. Symptoms typically occur in the early phase of illness and subside about 6 to 10 days after the onset of primary infection but may persist for weeks. Overlap of clinical symptoms of EBV with those of other infectious diseases has made this approach unreliable. In many hospital settings, patients suspected to have active EBV infection are routinely subjected to serologic test commonly referred to as 'heterophil antibody or monospot test'. Positive mono spot test accompanied by increased total number of lymphocytes, and existence of more than 10% atypical lymphocytes in blood normally confirms the presence of the disease. Several antibody tests are also available for determination of past or recent EBV infection. Current/ recent infections can be confirmed by presence of IgG antibody to the viral capsid antigen (VCA) and antibodies to the nuclear antigen (EBNA). Past infections on the other hand are confirmed by presence of IgM antibody to the viral capsid antigen (VCA). Just like physical examination, serologic tests can be unreliable since false-positive and false-negative may be found in a few patients (CDC, 2006).

Quantitative Real-Time Polymerase chain reaction (qRT-PCR) test for EBV DNA is a highly sensitive, specific and quantitative diagnostic approach. Since its advent, it has become an important tool for early diagnosis of EBV-associated cancers. Stevens *et al.* (2001 and 2005) have explained that a cutoff value of 2,000 EBV DNA copies /ml of whole blood clearly demarcates patients with EBV-associated disorders from healthy EBV carriers. Aalto *et al* (2007) on the other hand has discussed that individuals with EBV DNA levels $\geq$ 50 000 copies/mL are considered to have life-threatening EBV infection. Stevens *et al* in 1999 suggested that a viral load of up to 120,000 copies/mL may indicate a case of non-Hodgkin's lymphoma in Aids patients while elevated viral loads of up to 4,592,000 copies/mL of blood may indicate cases of Burkitt lymphoma. Although qRT-PCR is highly reliable, it is not widely used in resource-limited settings because of the high operational cost and training required.

### 1.2.5. Treatment of EBV infection

Although many antiviral drugs such as acyclovir have been shown to be potent inhibitors of EBV replication in cell culture, there is still no known cure or specific treatment of patients infected with EBV. A number of reasons have been postulated to explain the failure of these drugs. To start with, in acute EBV infections, symptoms are insidious in onset. The insidious onset together with a long incubation period of about 4 to 6 weeks results into delayed diagnosis of the disease. Secondly, since the virus is shed in saliva, levels of antiviral drugs achieved in the oropharynx after oral drug administration may not be enough to suppress virus titres in saliva. The third reason is that most clinical signs of this infection are due to immunopathogenic responses to EBV-infected cells and not due to viral cytopathology in infected tissues (Gershburg & Pagano, 2005). Treatment consists of measures to help relieve symptoms and keep the body as strong as

possible until the virus clears up. Such measures include medications to treat symptoms, plenty of drinking fluids and abundant rest.

Nevertheless, certain medicaments that include antiviral therapy coupled with immunomodulatory drugs are used. Two groups of drugs have been shown to be strong candidates for treatment of EBV. The first group comprises of compounds that target the viral DNA polymerase. These include acyclic nucleoside analogues (acyclovir, ganciclovir, penciclovir); acyclic nucleotide analogues (cidofovir and adefovir); pyrophosphate analogues; and 4-oxo-dihydroquinolines. The acyclic nucleotide analogues consist of compounds that are potent inhibitors of EBV replication. They include maribavir, β-L-5-iododioxolane uracil and indolocarbazole NIGC-I. Maribavir acts by directly or indirectly inhibiting viral protein kinases. It also interferes with nuclear activity of virions during maturation. Indolecarbazoles on the other hand causes slight inhibition of EBV BGLF4 protein (Gershburg & Pagano, 2005).

## 1.3. Problem statement

Epstein Barr virus is known to have a bearing on the occurrence of a number of syndromes and malignancies especially among children and adolescents. However, the viral-prevalence of EBV and its association with acute febrile illness have not been adequately studied in Kenya. The present study was therefore conducted to catalogue the viral-prevalence and viral loads of EBV in patients with acute febrile illness in Kenya.

## 1.4. Justification and significance of the study

EBV is a potential cause of acute febrile illness. It is however not among the differential diagnoses of acute febrile illness in Kenya, an indication that cases of AFI associated with EBV infection are overlooked. It is therefore important to describe EBV infection in acute febrile

cases as this will generate data and information that may find applicability in better management and treatment of patients with AFI.

## 1.5. Objectives

### General objectives

- To investigate Epstein Barr virus infection in patients presenting with acute febrile illness

### Specific objectives

1. To detect Epstein-Barr Virus and determine viral loads in patients with acute febrile illness
2. To determine EBV infection rates and viral load levels among age groups in patients with acute febrile illness
3. To examine the association between clinical characteristics and EBV viral load levels in patients with acute febrile illness
4. To determine whether exposure to malaria has an effect on EBV prevalence and/or levels of circulating EBV viraemia in patients with acute febrile illness

## 1.6. Hypothesis

Null hypothesis: Epstein Barr virus is not a common infection among patients with acute febrile illness in Kenya

# CHAPTER TWO: MATERIALS AND METHODS

## 2.1. Study design

### 2.1.1. Study site and subject recruitment

This study was a hospital based cross-sectional study. 2107 patients presenting with fever of unknown origin (defined as temperature $\geq 38\ ^0$C) were recruited from eight healthcare facilities located in the Lake Victoria Basin: Kisumu district hospital, New Nyanza provincial hospital and Alupe sub-district hospital; Kisii highland (Kisii district hospital), Semi arid areas: Marigat district hospital, Iftin sub-district hospital, Garissa police line clinic, and coastal region (Malindi district hospital). These healthcare facilities serve both urban and rural areas. Figure 3 is a map of Kenya showing the location of the eight healthcare facilities from which acute febrile patients were recruited. Blood specimen was drawn from each patient on the day of recruitment, and was temporary stored in liquid nitrogen dry shippers before being transported to the WRP/KEMRI research laboratory in Kondele, Kisumu where the laboratory assays was performed.

### 2.1.2. Ethical considerations and inclusion criteria

Eligible subjects were voluntarily enrolled and the study protocol was approved by the Ethical Review Committee of the Kenya Medical Research Institute, Nairobi (SSC #, 1282) and the Walter Reed Army Research Institute of Human Use Research Committee (WRAIR # 1402), Silver Spring, Maryland, USA (Appendix 1). Volunteers were included in the study upon signing and dating an informed consent (Appendix 2). Persons of any gender aged 1 year or older and having a fever (body temperature $\geq 38^0$C) were eligible to participate. Subjects not willing or unable to consent were excluded from participation. For children less than 18 years, the consent

was provided by the parent or guardian. For patients able to give only oral consent, a witness was allowed to sign the consent form on their behalf.


**Figure 3.** A map of Kenya showing sites from which blood samples of acute febrile patients were collected.

## 2.2. Sample collection

Following the consent process, 2 mL of EDTA whole blood was obtained by venipuncture. The samples were then aliquoted into multiple 1.8 mL tubes and temporary stored in liquid nitrogen dry shippers at the collection sites before being transported to the WRP/KEMRI research laboratory in Kondele, Kisumu. On arrival at the WRP/KEMRI research laboratory, the samples were transferred to a -80 $^{0}$C freezer until required. For use, samples were thawed to room temperature and kept on ice during the processing period.

## 2.3. DNA isolation

DNA was isolated using QIAamp MinElute Virus Spin Kit (QIAGEN sciences, Maryland, USA) in accordance with the manufacturer's recommendation. Briefly, 200 µL of blood was added to 25 µL of protease in order to deproteinize the sample. Cell lysis was accomplished upon addition of 200 µL of lysis buffer followed by a 15 seconds pulse vortexing and a 56 °C incubation for 15 minutes. The deproteinised lysed DNA preparation was then purified on QIAamp MinElute column by addition of 250 µL of absolute ethanol followed by centrifugation at 6000 *x g* for 1 minute. This was followed by a series of 'wash and spin' involving 500 µL of wash buffers and absolute ethanol, in that order, at a centrifugation speed of 6000 *x g* for 1 minute for each wash. To completely dry the membrane inside the column, a full speed centrifugation at 20,000 *x g* was performed for 3 minutes. Finally, the purified DNA was eluted from the column by addition of 100 µL of elution buffer and stored at -20 $^{0}$C until required. Two microliters of eluate was used for each PCR. This amount corresponded to 4 µL of the original whole blood sample.

## 2.4. Detection of EBV and determination of viral load

A BALF5 gene sequence from the EBV was amplified by a TaqMan- qPCR on an Applied Biosystems' 7300 sequence detection system (Foster City, CA, USA). The primers and experimental procedures used to detect and quantify the target gene have been described elsewhere (Kimura *et al.*, 1999). The forward primer had the sequence 5'-CGGAAGCCCTCTGGACTTC-3' while the reverse primer had the sequence 5'-CCCTGTTTATCCGATGGAATG-3'. The TaqMan probe had the sequence 5'-TGTACACGCACGAGAAATGCGCC-3' and was labeled at the 5' end with a FAM dye and at the 3' end with a TAMRA dye. These primers and probe were purchased from Applied Biosystems (Foster City, CA, USA). The reaction was performed in a 25 µL reaction mixture which consisted of 12.5 µL TaqMan (1x) universal PCR master mix (Applied Biosystems, Foster City, CA, USA), 0.5 µL of 0.2 µM of forward primer and reverse primers, 0.25 µL 0.1 µM of probe, 2 µL of DNA sample, and PCR grade water. Amplification was performed with the following conditions: $95^0$C for 10 minutes, and 45 cycles consisting of 95 $^0$C for 15 seconds followed by 60 $^0$C for 1 minute.

A sample was considered positive if the threshold cycle ($C_T$) exceeded the $C_T$ values of negative control samples (Fig 4 panel A). The positive control consisted of a plasmid EBV DNA (12,500 copies/ µL) that contained the BALF5 gene sequence. A standard curve from the $C_T$ values obtained from 10-fold serial dilutions of the plasmid DNA was constructed for each run. A plot of the log of initial target copy number for a set of standards versus $C_T$ should give a straight line (Higuchi *et al.*, 1993). Figure 4, panel B shows a typical example of a standard curve. It is from this standard curve that copy numbers of the unknown samples were calculated by interpolation of the experimentally determined $C_T$. The instrument software (7300 System Sequence Detection

Software version 1.3, Applied Biosystems, Foster City, CA, USA) performed the whole process of calculating the $C_T$ values, preparing a standard curve, and determining the copy numbers of the samples.

To control for cross-contamination, two samples, namely water for no template control and EBV negative human sample for non-target template were also included in the assay. The amplification results were valid only when the positive control generated the acceptable $C_T$ value while the negative controls (NTC and hDNA) remained undetected. DNA concentrations in copies/mL were then calculated according to the following equation:

$$whole\,blood\,copies/mL = copies/rxn \times {}^{1}\!/_{4}\,mL\,of\ sample/rxn$$

## 2.5. Data management and analysis

Numerical data were expressed as proportions and compared using Pearson's Chi-square or Fisher's exact tests as appropriate. Continuous data were expressed as medians with interquartile ranges. Comparison of continuous data from patient groups was carried out using Kruskal-Wallis test or Mann-Whitney U test as appropriate. Significance levels were set at 0.05 while confidence intervals were held at 95%. Statistical analyses were performed using GraphPad Prism 5.

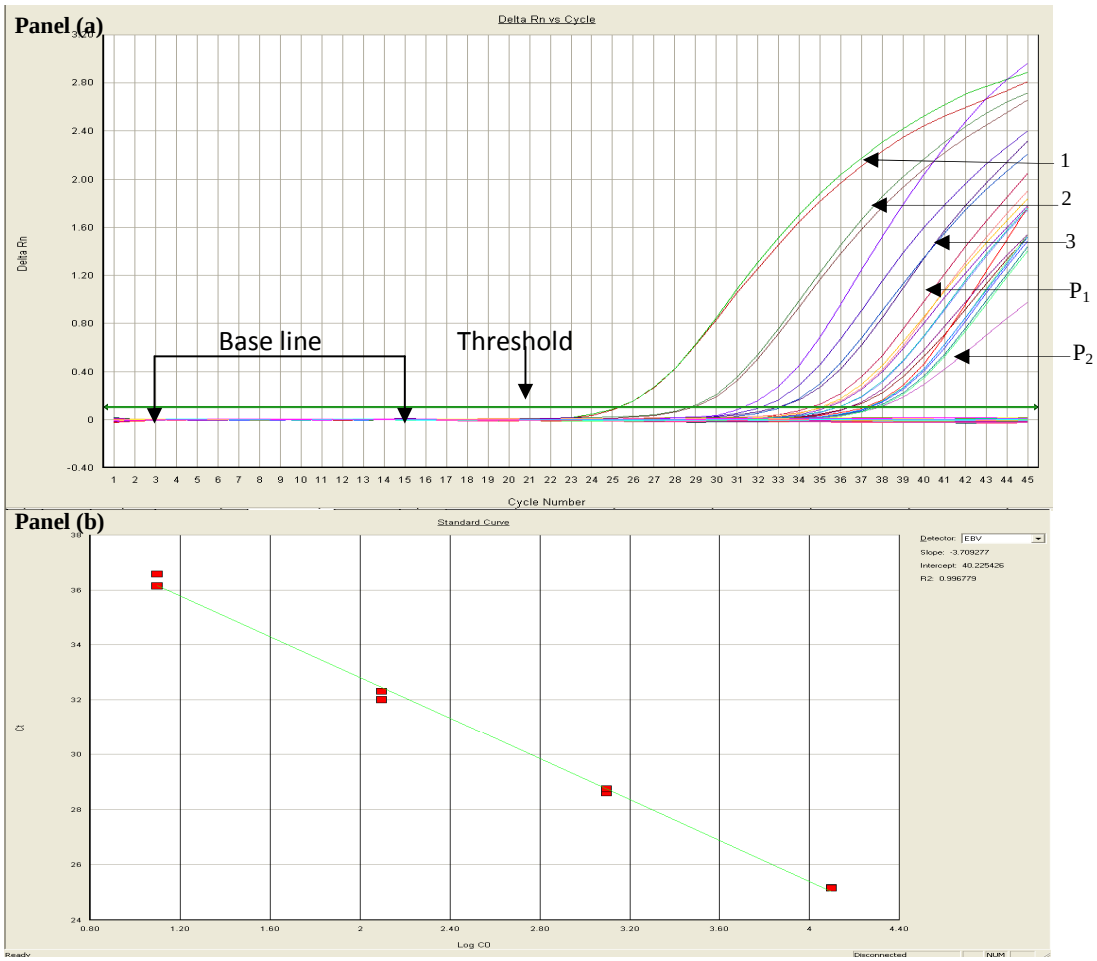


**Figure 4.** Panel A: Standard curves generated from plasmid of known concentration. Panel B: Amplification of the BALF5 EBV gene in 10-fold dilutions. The logarithm of the change in fluorescence, Rn, is plotted against the cycle number for the EBV gene.

# CHAPTER THREE: RESULTS

## 3.1. EBV prevalence in patients with acute febrile illness

This study was conducted on 2,107 AFI patients between the period of September 2008 and April 2013. For patients where demographic data was available, 1,033 were male (51.1%) and 989 were female (48.9%). The range of ages was 1 year to 80 years, and the median age was 5 years. The overall viral prevalence of EBV was 29.1% (614 of 2107 febrile cases), which included 309 male (29.9%) and 279 female (28.2%). There was no significant difference between EBV prevalence in males and females ($X^2$= 0.6300, df = 1, p = 0.4274, Fisher's exact test). EBV prevalence was age dependent, being highest in the 0-4 year olds (37%) compared to 25% in the 5-9 year-olds , 20% in the 10-14 year-olds, and 21% in those above 15 years ($X^2$= 51.36 , df = 1, p< 0.05, Chi-square test) (Figure 5, panel A). EBV viral loads ranged between 52 to 7.19 x$10^6$ copies/mL (median, 4,699 copies/mL) and were also age dependent. The highest viral load was recorded in the 0-4 year-olds (median 5,368, interquartile range 1,958-16,451) compared to the 5-9 year-olds (median 2,783 copies/mL, interquartile range 1,358 – 8,385 copies/mL) (U= 18360, p = 0.0008, Mann Whitney test) (Figure 5, panel B). 72% (443/614) of the patients with EBV had viral load above the 2,000 copies/mL, the cutoff value for EBV-associated disease (Stevens et al., 2001 and 2005), and were therefore considered as having active EBV infection. More than half of patients (241/443) with EBV viral load above this cutoff value were under 5 years of age (54.4%), compared to 89 (20.1%) in the age of 5-9 years, 15 (3.4%) in the age of 10-14 years, and 72 (16.3%) above 15 years of age.

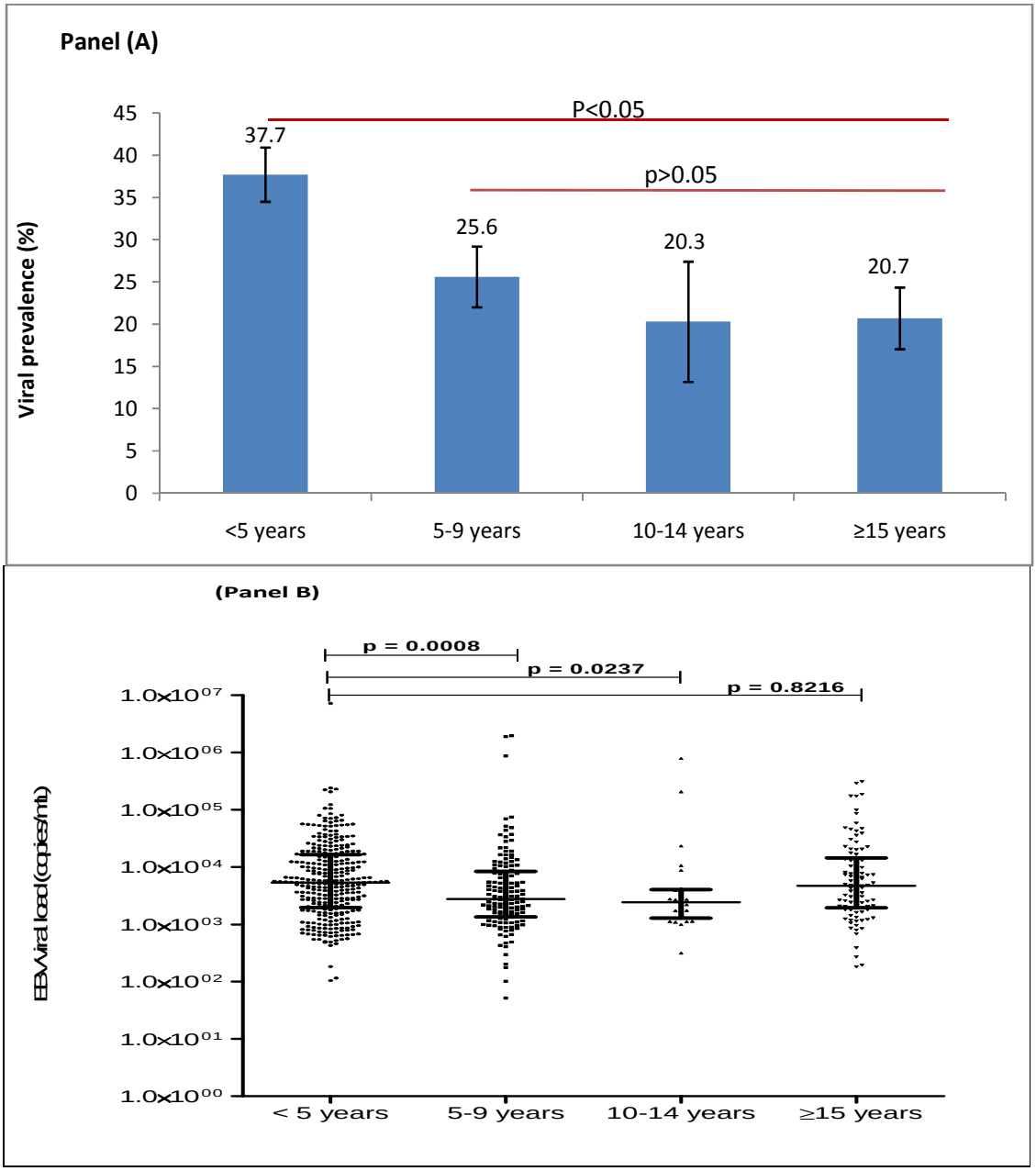


**Figure 5.** Panel A: EBV prevalence in different age groups of patients with acute febrile illness. Prevalence in the <5 year-olds were significantly higher than in other age groups ($p<0.05$). Panel B: Comparison of EBV viral loads between age groups. Patients without demographic data are not shown, and thus are excluded in analysis involving age.

As shown in Figure 6, regional EBV infection rates were highest in Lake Victoria region (36.8%) and coastal region (29.6%) compared to Kisii highland (23.7%), and semi-arid areas (22.2%). This difference was statistically significant ($p< 0.05$, Chi-square test) (Appendix 3). The proportion of patients with viral load above the 2000 copies/mL cut off value for probable EBV associated disorders was also higher in the coastal region (38/45, 84%) and the L. Victoria region (246/322, 76%) than in the Kisii (75/116, 65%) and semi arid region (84/131, 64%) ($X^2$= 13.72, df = 3, $p< 0.05$, Chi-square test) (Fig 7) (Appendix 4).

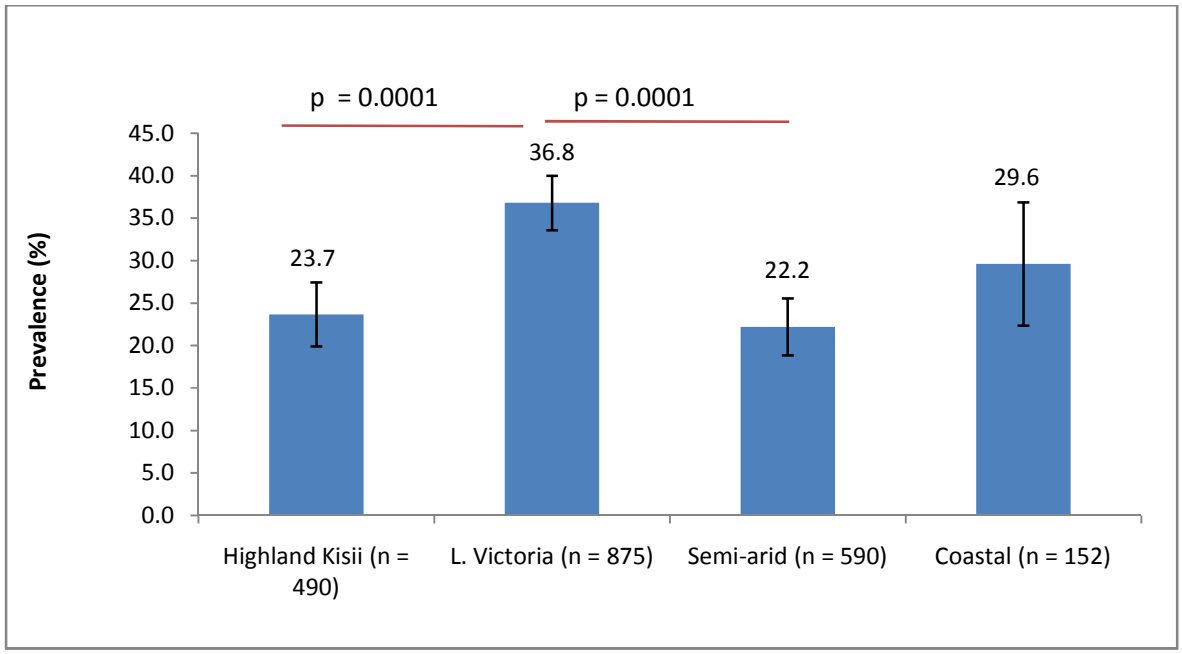


**Figure 6.** Regional EBV infection rates were highest in the Lake Victoria basin and coastal region compared to Kisii highland and the semi-arid region.

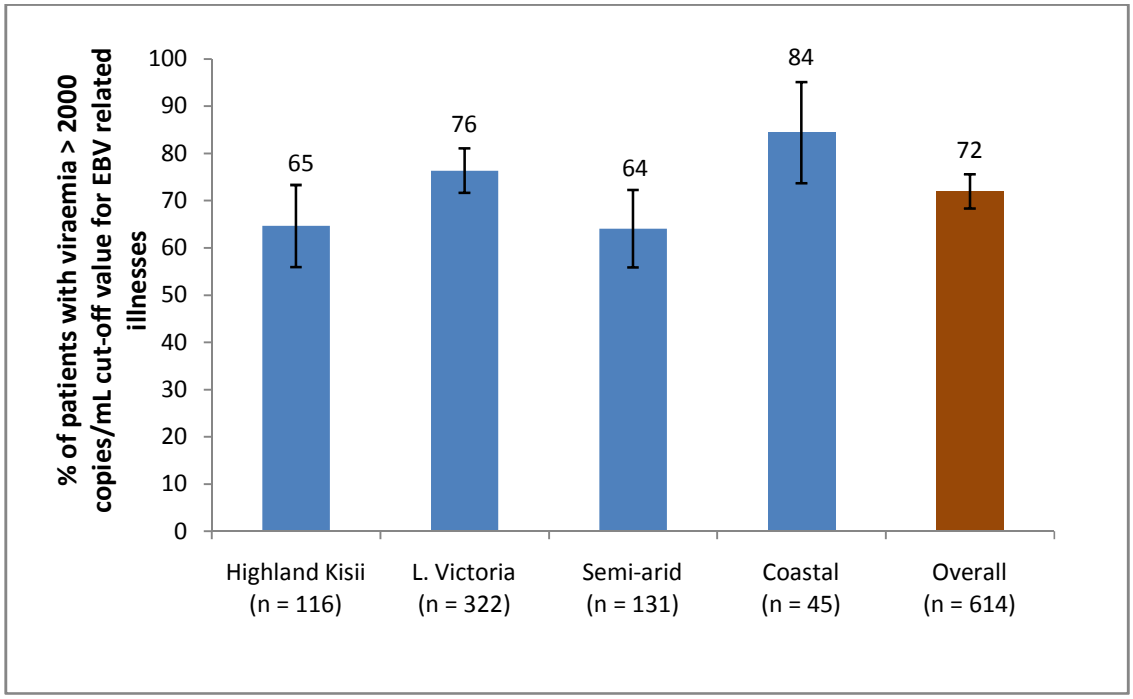


**Figure 7.** Proportion of AFI patients with EBV viraemia above > 2000 copies/mL in four regions of Kenya. The coastal and L. Victoria regions had significantly higher cases of AFI with viral load > 2000 copies/mL than highland Kisii and semi arid areas.

## 3.2. Association of EBV viral load levels and presenting signs

The most commonly reported clinical signs of AFI patients with or without EBV were headache, cough, chills, runny nose, joint aches, muscle aches, abdominal pain and sore throat. This study sought to determine the influence of viral load levels on the occurrence of these symptoms. All individual clinical signs assessed, with the exception of runny nose, abdominal pain, and sore throat did not show significant association with change in EBV status (Table 1). A higher percentage of cases reporting with runny nose were positively associated with increasing viral loads ($p = 0.0006$). Patients with complaints of abdominal pain were significantly higher in EBV sub-clinical group (i.e. viral load of between 1 and 2000 copies/mL) than in those negative for

EBV and those with viral load ≥2,000 copies/mL. On the other hand, cases reporting with sore throat reduced significantly with an increase in the levels of viral load (p = 0.0024) (Table 1).

**Table 1.** Association of EBV viral load level with AFI clinical symptoms. The table shows the trend in the occurrence of symptoms (%) with respect to change in viral load levels

| **Characteristic** | **EBV Negative** | **Sub-clinical (1 – 1,999 copies/mL)** | **Clinical (≥ 2,000 copies/mL)** | **$X^2$-value (df=2)** | **p-value** |
|---|---|---|---|---|---|
| Symptom | | | | | |
| Chills | 48.8 | 52,1 | 42.2 | 3.27 | 0.1947 |
| Cough | 54.4 | 57.9 | 56.9 | 1.07 | 0.5852 |
| Sore throat | 24.0 | 15.8 | 15.8 | 12.16 | 0.0024 |
| Headache | 59.6 | 59.1 | 43.6 | 1.93 | 0.3778 |
| Runny nose | 43.4 | 50.9 | 52.8 | 14.73 | 0.0006 |
| Abdominal pain | 39.1 | 42.7 | 29.8 | 7.85 | 0.0203 |
| Joint aches | 36.1 | 33.3 | 27.5 | 1.18 | 0.5452 |
| Muscle aches | 21.2 | 23.4 | 17.2 | 1.97 | 0.3837 |
| Signs | | | | | |
| Mean temperature, °C, ± SD | 38.8 ± 0.59 | 38.9± 0.59 | 38.9 ± 0.63 | $F_{(2,1947)}$=0.527 | 0.5874 |
| Mean illness duration, days , ± SD | 3.6 ± 2.61 | 3.6 ± 2.32 | 4.0 ± 2.62 | $F_{(2,1934)}$=0.624 | 0.0536 |

Higher percentage of patients with runny nose was reported in the clinical group while the proportion of patients with abdominal pain was highest in the sub clinical group than those negative for EBV and those in the clinical stage EBV infection ($p < 0.05$). Sore throat was negatively associated with EBV infection ($X^2$= 12.16, df = 2, p = 0.0024, Chi-square test).

## 3.3. Effect of malaria exposure on EBV prevalence and viral load

To evaluate whether exposure to malaria impacts prevalence of EBV, study participants were divided into two groups. Group one consisted of patients residing in areas considered to have intense malaria transmission (holoendemic malaria group), and included patients recruited from Kisumu district hospital, New Nyanza provincial hospital, Alupe sub-district hospital, and Malindi district hospital. Group two consisted of patients residing in areas of sporadic malaria exposure (Hypoendemic malaria group). Patients in this group were recruited from Kisii district

hospital, Marigat district hospital, Iftin sub-district hospital, and Garissa police line clinic. As shown in Figure 8, prevalence of EBV was significantly higher in the holoendemic malaria group (35.7%) compared to the hypoendemic malaria group (22.9%) ($X^2$= 42.19, df = 1, p = 0.0001, Chi-square test). Within the holoendemic areas the prevalence was highest (42.4%) in children below 5 years when compared to other older age groups; 5-9 years (32.3%), 10-14 years (20.0%), and ≥15 years (22.3%) ($X^2$= 26.58, df = 3, p<0.05) (Fig. 9, panel A). Unlike the holoendemic malaria group, there was no significant difference in EBV positivity between age groups in the hypoendemic malaria group ($X^2$= 4.626, df = 3, p > 0.05) (Fig. 9, panel B).

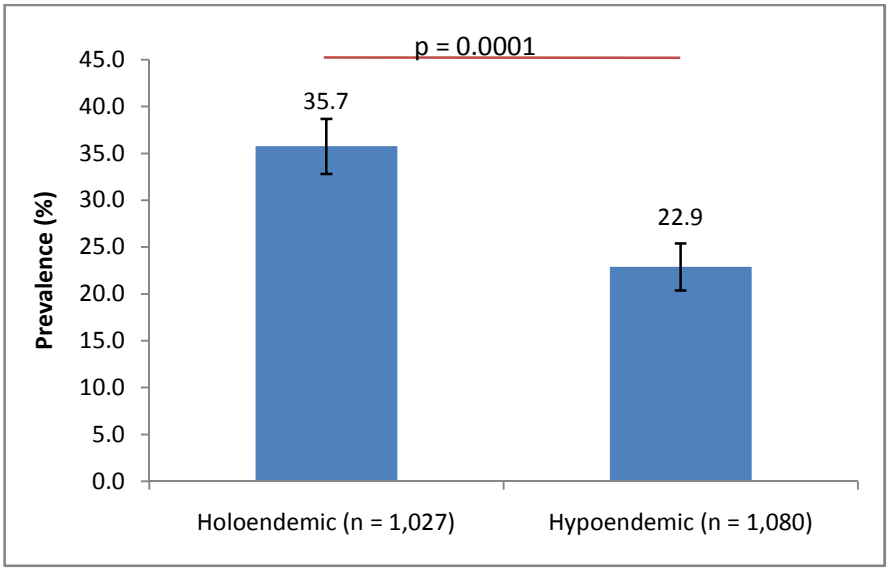


**Figure 8.** Bar graph showing prevalence of EBV infection in holoendemic and hypoendemic malaria areas

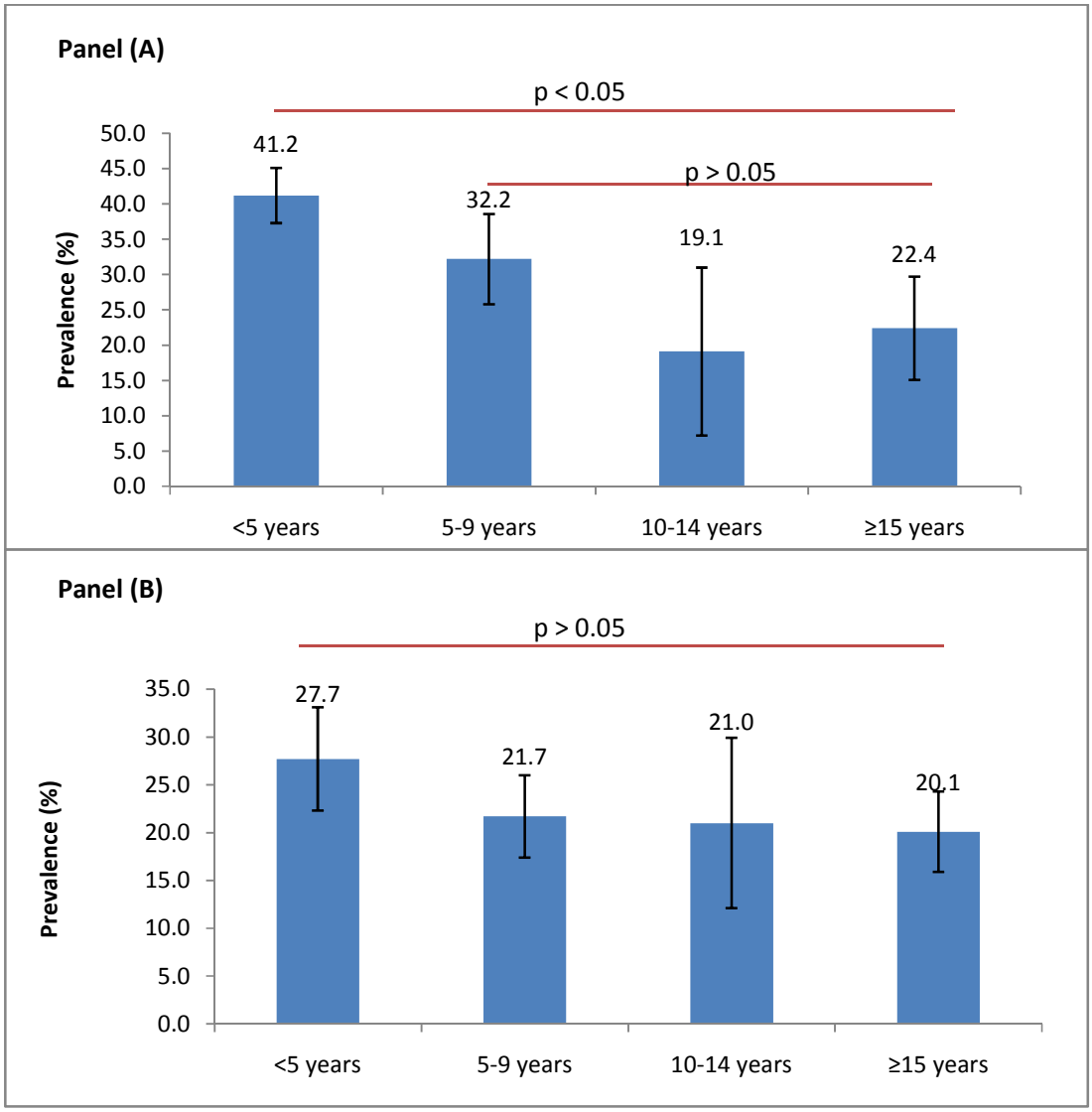


**Figure 9**. Panel A: EBV infection rates in different age groups in areas with intense malaria exposure (holoendemic malaria). Panel B: EBV infection rates in different age groups in areas with relatively low frequencies of malaria exposure (hypoendemic malaria).

We next evaluated whether coinfection of EBV and malaria results into an increase in the level of circulating viral load. As shown in Figure 10, study participants coinfected with EBV and malaria had significantly higher viral load (median 5,329 copies/mL, interquartile range 2,455-

14,596) compared to patients with EBV alone (3,655 copies/mL, interquartile range 1,443-12,285) (U= 25170, p= 0.004, Mann Whitney test) (Fig. 10).

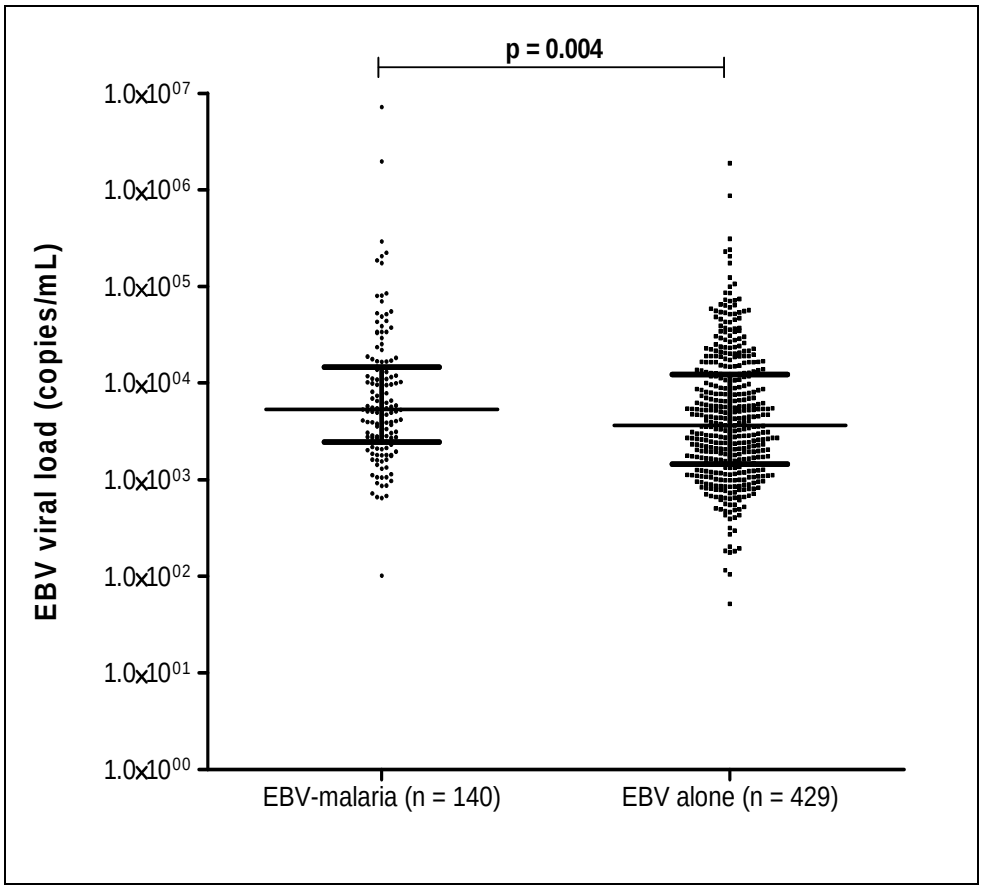


**Figure 10.** Febrile patients co-infected with EBV and malaria had a higher viraemia compared to individuals infected with EBV alone.

Levels of circulating EBV virus with regard to age were also compared in the two groups. Statistically significant difference in viral load was observed only in the 5-9 years age group, where those with EBV-malaria co-infection had higher viral load (median 3,849 copies/mL, interquartile range 1,935-11,715) compared to those with EBV alone (median 2,243 copies/mL, interquartile range 1,075-6,508) (U= 1354, p= 0.008, Mann Whitney test) (Fig.11).

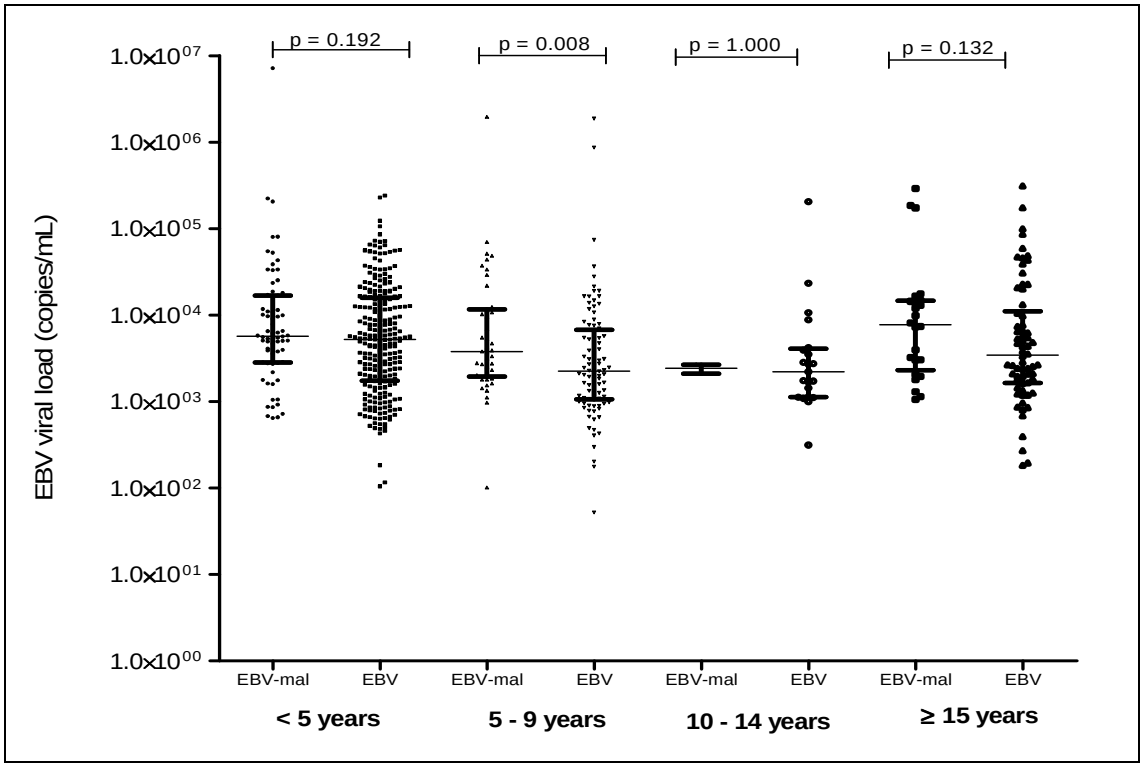


**Figure 11.** Comparison of viral loads between age groups among patients with EBV-malaria co-infection (EBV-mal) and those with EBV alone

# CHAPTER FOUR: DISCUSSION

## 4.1. Summary of discussion

Acute febrile illness is a common cause of hospital admission and death in developing tropical and sub–tropical countries (Animut *et al.,* 2009; Pondei *et al.,* 2012). Making correct diagnosis and selecting effective therapy for AFI patients remains a public health challenge in many healthcare facilities, particularly in areas where diagnoses are made on clinical grounds (WHO, 2011). A wide array of diseases can present as AFI, ranging from malignancy, collagen vascular disease, miscellaneous non-infectious conditions, and infectious agents of viral, bacterial, and protozoal nature (Cunha, 2007; Chow & Robinson, 2011).

A clinically useful system to identify EBV and many other infectious agents as the cause of fever is laboratory testing. In Egypt, for instance, community-based studies show that most patients with AFI are diagnosed and managed clinically as having typhoid fever. These studies have found that without laboratory testing, it is difficult to distinguish typhoid fever from other causes of AFI such as brucellosis and rickettsial diseases (Crump *et al.*, 2003). In sub-Saharan Africa, the presumptive treatment for all fevers as malaria has been the adopted strategy, as recommended by the Integrated Management of Childhood Illnesses (Bojang *et al.,* 2000; Chandramohan *et al.*, 2002). However, recent studies have found out that majority of these unexplained fevers are of non-malarial origin (WHO, 2011). The advent of real time qPCR has revolutionized diagnosis and monitoring of many diseases. Although it is an expensive technique in terms of operational cost, it is generally reliable for early diagnosis of EBV disorders as well as monitoring response to treatment. Its sensitivity, specificity, speed, and ease of handling has made it an important tool for clinical diagnosis (Leung *et al.*, 2002; Lay *et al.*, 2010). Generally, a viral load of greater than 2000 copies per milliliter of whole blood may result into EBV-

associated diseases (Stevens *et al.,* 200 and 2005). According to Aalto *et al* (2007), individuals with EBV DNA levels ≥ 50,000 copies/mL are considered to have life-threatening EBV infection. A viral load of up to 4,592,000 copies/mL of blood may indicate cases of Burkitt lymphoma while elevated viral loads of up to 120,000 copies/mL may indicate a case of non-Hodgkin's lymphoma in Aids patients (Stevens *et al.*, 1999). In this study, viral prevalence, load and their association with patient's characteristics and malaria were determined by qPCR in AFI patients who presented in various hospitals in Kenya.

#### 4.1.1. EBV infection rates in febrile patients

The overall prevalence of EBV among the evaluated febrile patients was 29%. As has been observed before (Norzuriza *et al.,* 2008), there was no gender influence on prevalence. Infection rates were highest in the age group <5 years (37%), and reduced thereafter with increase in age. These data illustrate high EBV exposure among children below the age of 5 years, which is consistent with a study by Figueira-Silva and Pereira (2004) who reported that the infection occur more frequently at a younger age in children from families with low socioeconomic status in the state of Espírito Santo, Brazil. A seroepidemiological study conducted in Bangladesh showed that 42% of infants had antibodies to EBV by the age of 1 year, and a significant rise in the percentage of sero-positives was observed between 0-1- and 1-2-year-old children (Haque *et al.*, 1996). In Malaysia, 100% of older individuals (> 60 years) were found to be carriers of EBV, reducing to 98.6% among 21-40 year-olds, 96% in 41-60 year-olds and then to 77.5% in the 0-20 year olds (Norzuriza *et al.*, 2008). Additionally, a study in Japan documented high infection rates of EBV in early years of childhood. The study reported that > 90% of 5-9 year-old children had EBV antibodies (Takeuchi *et al.*, 2006).

In the developing countries, the poor socio economic status, crowding especially in public schools, and the practice of pre-chewing food for infants have previously been associated with acquisition of EBV (Sumaya *et al.*, 1975; Biggar *et al.*, 1978; Crowcroft *et al.*, 1998). Although acquisition of EBV infection in the preadolescent stage has been shown not to result into infectious mononucleosis, recent data suggest that this early acquisition is harmful. In southern China, for instance, early infection has been associated with occurrence of nasopharyngeal carcinoma (Chang & Adami, 2006). Occurrence of multiple sclerosis, a condition of the nervous system, has also been linked to early acquisition of primary EBV. A study by Pohl and colleagues (2006) showed that a significantly higher proportion of children with multiple sclerosis had been infected with EBV than matched controls, and that the infection had been acquired sometime before the diagnosis of multiple sclerosis

In this study, viral load data of 443 out of 614 EBV infected individuals (72%) had a viral load above the 2000 copies/mL of blood (Figure 7), a cutoff threshold for EBV related illness (Stevens *et al*., 2001 and 2005). This is contrary to previous studies that have indicated that infection during early childhood, especially in countries of low socioeconomic status, is largely asymptomatic (Figueira-Silva & Pereira, 2004; Snider *et al*., 2012). From this data, it is of the opinion that, 21% (443/2107) of the AFI patients studied probably came to the hospital because of EBV related infections. The highest burden associated with symptomatic EBV infection (241 out of the 443) was borne by children <5 years old (54%). This proportion was significantly higher compared to the other three age categories i.e. 20% for the 5-9 year-olds, 3% for 10-14 year-olds, and 16% for the > 15 years. These findings again contradict reports which have persistently claimed that symptomatic primary EBV infection as a rare phenomenon in early childhood (Figueira-Silva & Pereira, 2004; Haahr *et al.*, 2004; Junker, 2005; Tarbouriech *et al.*,

2006; Hjalgrim *et al.*, 2007). These studies document that symptomatic EBV infection or infectious mononucleosis is a disease of the adolescents, occurring when they get primary infection through the intimate kissing.

Socio-economic factors have been shown to influence sero-prevalence rate of EBV (Figueira-Silva and Pereira, 2004). Thompson & Kurzrock (2004) documented that, in addition to socio-economic differences, geographical location and hygiene play significant role in the occurrence of EBV. In regions such as Africa, Southeast Asia, and Latin America where substantial proportions of the population are of low socioeconomic status, EBV prevalence is near 100% by early childhood (Figueira-Silva & Pereira, 2004; Haahr, *et al.*, 2004; Junker, 2005). In the current study, infection rates were relatively higher in the Lake Victoria basin at 37%, 30% in the coastal region, 24% in the highlands of Kisii and were lowest in the semi-arid region, at 22%, ($p< 0.0001$) for difference between Lake Victoria region, Kisii highlands and semi-arid region. In addition, the Lake Victoria and coastal regions had the highest cases of febrile patients with EBV viral load $\geq$ 2000 copies/mL cutoff value compared to Kisii and semi-arid areas. The possible explanation for this higher EBV prevalence in L. Victoria and the coast could be the existence of prevalent malaria and HIV in these regions (NASCOP, 2005; DOMC, 2009) which possibly lead to immunosuppression in individuals that would subsequently succumb to EBV infection.

### 4.1.2. Association of EBV viral load levels with patients' characteristics

Clinical features of symptomatic EBV infection are usually non-specific. However, the severity of these clinical signs and symptoms has been shown to vary with the status of EBV infection. In most cases, a triad of fever, lymphadenopathy, and pharyngitis are reported in acute EBV infection. Other symptoms may include abdominal discomfort, palatal petechaie, rash,

splenomegaly, hepatomegaly, jaundice, and periorbital oedema. Prior studies on various viral diseases such as HIV/AIDS, Burkitt lymphoma, hodgkins lymphoma, nasopharyngeal carcinoma, and Post-transplant Lymphoproliferative Disorder (PTLD) have suggested that viral loads in whole blood or plasma as measured by quantitative PCR mirrors clinical status of the diseases (Yang *et al.,* 2006; Asito *et al.,* 2010; Gulley & Tang, 2010; Hohaus *et al.,* 2011). In order to find any clinical manifestation that could predict elevated EBV viral load and help in clinical decision-making, the current study attempted to find the association between viral load and various AFI clinical features. Increasing number of complaints of runny nose was significantly associated with higher viral load. Sore throat and abdominal pain on the other hand were negatively associated with the increase in viral load. Other clinical complaints such as chills, cough, headache, muscle aches, joint aches, body temperature, and illness duration did not show any significant trend with changes in the levels of viral load. Although runny nose was significantly associated with change in viral load levels in this study, more investigations need to be carried out to ascertain this. A number of other reports have suggested that the levels of circulating EBV do not necessarily reflect the occurrence of clinical manifestations of EBV associated disorders. Instead, they have associated the clinical features with the intensity of immune reactions elicited by EBV, especially the activity of cytotoxic T cells (Greshburg & Pagano, 2005; Cameron *et al.,* 2006; Drebber *et al.,* 2006; Hara *et al.,* 2006; Hong *et al.,* 2012). It therefore may not be appropriate to link runny nose to change in levels of EBV as depicted in this study.

### 4.1.3. Effect of malaria exposure on Epstein - Barr virus infection

Studies on EBV in Kenya are mostly focused on the life-threatening endemic Burkitt lymphoma (eBL), a common pediatric cancer accounting for 70% of cancer related childhood mortality in

equatorial Africa (Moormann *et al.,* 2011). This is solely because EBV and malaria parasites have been found to be the major cofactors in the pathogenesis of this lymphoma. It is however not known how these pathogens interact to cause eBL (Moormann *et al.,* 2005; Moormann *et al.,* 2007; Chattopadhyay *et al.,* 2013). Since there is currently no documentation on the prevalence of EBV in Kenya, little is known on the effect of malaria on the occurrence of EBV infections. Therefore examining whether malaria endemicity and infection influence the prevalence of EBV infection as well as the level of circulating viral load is an important issue in this wake of increasing cases of EBV associated illnesses. The present study has revealed that EBV infection is more prevalent in areas of intense malaria transmission than areas of sporadic malaria transmission. The results also show that in holoendemic malaria areas, higher EBV infection rates are more likely to occur in children below the age of five years than would occur in older age groups. This however seems not be true in areas of sporadic malaria transmission.

Clearly, viral load was higher in EBV-malaria co-infected group compared with EBV infected group without concurrent malaria infection. This phenomenon was also particularly observed among the age groups, with cohorts of EBV-malaria co-infected group showing increased viral loads than their counterparts in EBV infected group without concurrent malaria infection. This was much more pronounced in the 5-9 years age group, a finding that corroborates with what has been previously published as the age with the highest risk of developing Burkitt lymphoma (Morrow *et al.,* 1976; Mwanda *et al.,* 2004) and thus calls for critical investigation in the future. Our results agree with earlier report by Lam *et al* (1991) who found that children with acute malaria had a higher frequency of EBV-infected B-cells than children who had recovered from malaria. Similarly, a study by Moormann *et al* (2005) documented that children between the age

of 1 year and 4 years living in areas with higher malaria exposure had elevated EBV viral loads when compared to those living in areas with relatively low exposure to malaria.

Thus, the finding in the current study and those reported above could suggest that the possible cause of the increased EBV prevalence and elevated levels of EBV in individuals with concurrent EBV and malaria infections may be due to the activities of malaria parasite in the host. Studies elsewhere have shown that malaria infection induces apoptosis (Toure-Balde *et al.,* 1996). This parasite-induced apoptosis would contribute to reducing the immune response directed to other infections, by increasing the fragility of potential effectors cells. The malaria antigen-induced apoptosis may also alter the lymphocyte composition in the peripheral blood (Toure-Balde *et al.,* 1996) which, in the case of co-infection with EBV, may result into increased viral replication. Rose *et al.* (2001) and Jayasooriya *et al.* (2012) have expounded further by suggesting that EBV-specific T-cell immunity become impaired during malaria co-infection, either as a cause or consequence of enhanced EBV replication. This consequently results into a reduction in T-cell responses which, in turn, cause loss of viral control. It has also been suggested that malaria parasite has a tendency of inducing polyclonal B-cell expansion (Moormann *et al.,* 2005; Njie *et al.,* 2009*;* Korir *et al.,* 2012). Thus elevated viral loads among individuals living in areas with high intensity of malaria transmission, and those with concurrent EBV and malaria infection probably reflect an indirect expansion of EBV-infected B cells in their peripheral blood. It can therefore be said that the expansion of EBV-infected B-cells during malaria infection enhances an increased circulation of EBV virus in saliva for spreading to other human hosts which, in turn results into increased EBV prevalence.

There were several significant limitations to our study. First, the use of a hospital based cross-sectional study design meant that infection rates and virus burden were likely to be underestimated. This is because this approach captures only a fraction of patients with AFI that seek hospital care. Those who present to the health care centers and those that are willing to participate in such studies are often not fully reflective of the population at-large, which may lead to biases in age-dependent incidence rates. Therefore the prevalence rates presented here should be considered a conservative estimate of the true EBV prevalence among the febrile patients. Second, previous studies have shown that viral load measurements are affected by the method of DNA purification, the time taken for DNA to be isolated from whole blood and subsequent subjection to PCR, and the freeze thaw cycles a sample is subjected to. Riemann and colleagues reported that yield from manually extracted DNA was 57% higher than that of robotic systems (Riemann *et al.,* 2007). For optimal quantitation, it has been shown that DNA samples should be subjected to PCR within one to two weeks after extraction (Malcomson *et al.,* 1995; Lay *et al.,* 2010).

Lahiri and Schanbel (1993) have indicated that blood samples subjected to more than four freeze-thaw cycles become degraded. In this study, we used a silica-based column method (QIAGEN kit) that has been shown to yield highly purified DNA (Fafi-Kremer *et al.,* 2004; Schuurman *et al.,* 2005). However, delayed DNA isolation and amplification could have contributed to reduced DNA loads and positivity in our samples. Also, since most of the earlier samples we used were tested retrospectively, monitoring these conditions was not possible. Third, in the current study, one of our objectives was to determine the effect of malaria infection on the levels of circulating EBV. Since data for malaria parasitemia was lacking from study

participants, it was not possible to fully describe the influence of malaria parasite on EBV viraemia.

# CHAPTER FIVE: CONCLUSIONS AND RECOMMENDATIONS

## 5.1. Conclusions

This study has shown that about 30% of febrile cases in areas of study have detectable EBV infections and that about 25% would be classified as having symptomatic EBV infections. Children under the age of 5 years especially in areas with intense malaria exposure are at a higher risk of developing symptomatic EBV infection. The analysis of EBV infection with regard to geographical conditions has confirmed that local environmental conditions predispose to EBV prevalence and viral load levels in an individual. The study has also shown that the onset of runny nose in EBV infections may be attributed to elevated viral load. This may help in clinical decision-making. It can also be concluded that malaria impacts prevalence of EBV as well as elevated EBV viraemia, which may be explained in part due to *Plasmodium*'s ability to cause expansion of EBV infected B-cells and/or to compromise the number of functional EBV specific cytotoxic T-cells. This, in turn, results into increased availability of the virus in saliva for transmission. There is need to create more awareness among healthcare workers that other than malaria, other infectious agents, including EBV, can be important causes of acute febrile illness in Kenya.

## 5.2. Recommendations

I. The findings of this study show that within the 5-9 years age group, patients with concurrent EBV and malaria infections had significantly higher viral loads than those with EBV alone. Published data shows this is the age with the highest risk of developing Burkitt lymphoma (Morrow *et al*., 1976; Mwanda *et al*., 2004). It will be of great interest

to investigate whether this elevated viral load in EBV-malaria co-infections at this age bracket acts as a precursor to the development of Burkitt lymphoma.

II. Although this research has shown a significant trend between viral load levels and runny nose, a detailed analysis of the relationship between viral load level and clinical signs and symptoms is warranted in future studies.

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# APPENDICES

## Appendix 1: Informed consent form

**What is the study called:** Acute febrile illness surveillance in Kenya.

**What is this study about:** This is a research study where we are trying to find out the causes of illnesses in adults and children who have fever but no other signs of being sickness. We want to draw a small amount of blood and swab the inside of your nose and test it in the lab to see if we can find the germ that is causing your illness. Additionally the study is testing a new method of diagnosing malaria.

**Who is running the study:** The study is being run by Dr John Waitumbi from United States Army Medical Research Unit-Kenya (USAMRU-K)/Kenya Medical Research Institute, Nairobi, Kenya.

**Do I have to participate:** Participation in this study is voluntary. There is no penalty for refusing to participate. If you (your child) decide not to be in the study you (your child) may still receive medicine to help with your fever. If you start the study you (your child) may stop the study at any time. If you (your child) stop the study after we draw your (your child's) blood or take the swab from your (your child's) nose, we will destroy these samples so that no future testing can be done.

**Do I have other choices:** You have the option of not participating in the study and just continuing with the health care plan as directed by the health care provider at the clinic.

**What will happen to me if I participate in the study:** You will be asked some questions about where you live, your illness and any medications you may have taken recently. Then about 1-2 tablespoons of blood will be taken from a vein in your arm. The blood will be put into small tubes so we can test for germs that may be causing your illness. We will also take a swab from your nose to test for germs that may be causing your illness.

The information collected about your illness and where you live will be used by study team to determine your home's location on an electric map (called Global Positioning System -GPS) and assign it a unique house number. The GPS mapping provides information about how diseases spread in communities. By documenting the location of everyone's home, we can better understand how diseases spread in your community and can contribute to understanding the best way to monitor for emerging infections, outbreaks and epidemics.

**Are there any risks if I participate in the study:** There is the possibility of mild discomfort, bruising and very rarely infection at the site of needle injection.

We will protect all personal information we collect. You (your child) will be assigned a study number that will be used on all study documents. All study documents will be secured and will only be accessible to authorized study personnel.

**Are there any benefits from the study:** Because we are conducting this study at this health care facility, free malaria testing and treatment is available. If you want to, you can receive medication to treat your (your child's) fever.

Additionally, the study can lead to a better understanding of the causes of fever in Kenya and improve the medical care in your (your child's) area. Your hospital may learn about new or emerging diseases that cause fever. There are no direct benefits to you for participating in the study.

**Will there be any compensation for being in the study:** There is no compensation to volunteers for their participation. But, should you (your child) be injured as a direct result of participating in this research project, you (your child) will be provided medical care, at no cost to you (your child), for that injury. You (your child) will not receive any injury compensation, only medical care. You (your child) should also understand that this is not a waiver or release of your (your child's) legal rights.

**How long does the study last:** This study requires only completion of the questionnaire, one blood draw and nasal swab. There is no follow-up or further information needed. The questionnaire and other study procedures will take about 30 minutes.

**Who can participate in this study:** Anyone (including pregnant women) can participate in the study if you have a fever without a source after evaluation by the clinician. If there is an obvious source of infection causing the fever, like a skin infection or lung infection, then you (your child) should not participate.

**Who will be able to see my information or lab results:** Any information about you (your child) will remain confidential. Only the people involved in the study will be able to see your information. We will protect all your information. The information and specimens we collect may also be reviewed by representatives of the Ministry of Health, Kenya Medical Research Institute, the U.S. Army Medical Research and Materiel Command (USAMRMC), and the Walter Reed Army Institute of Research as part of their responsibility to oversee this research, for legal reasons or to investigate an outbreak. Federal Regulatory Agencies from United States and other local regulatory agencies may review the study records. In the event of an outbreak, the Kenya MoH will be provided with information about the location (and/or the individual) to investigate the outbreak of an illness that is considered reportable to the Kenyan MoH. Federal Regulatory Agencies from United States and other local regulatory agencies may review the study records.

Any report from this study will refer to you/your child only by a study identification number and not by a name. All blood samples, and nasal swabs collected will be labeled with a study identification number; no names will be used. The lab tests will take a long time to complete and will not be available to you or your health care provider to use in your care.

**What will happen to my blood:** Your (your child's) blood will be tested for things that could cause fever. Your blood will NOT be tested for HIV. A sample of your blood will be kept frozen in case we want to do more testing on it in the future. Testing may include developing new lab tests or looking for new or emerging diseases. These samples will be labeled with only your study number. They will be

secured in freezers at WRP/KEMRI facilities and only study investigators and their authorized staff will have access. All safeguards ensuring privacy that are in place during this study period will also continue to be in place for the long-term storage of samples. If samples are sent outside of Kenya for additional lab testing, no personal identifiers will be included.

If we do need to use the stored blood samples in the future we will first get permission from the Walter Reed Army Institute of Research (WRAIR) Institutional Review Board (IRB) and Kenya National Ethical Review Committee.

**Who can I contact about the study or my rights as a volunteer in this research study:** If during the course of this study, you have questions concerning the nature of the research or you believe you have sustained a research-related injury, you should contact:

Dr. John N. Waitumbi

USAMRU-K /Walter Reed Project, PO Box 54,

Kisumu

Tel. 0733 333 530 or +254 57 20 22 942

The Chairman of the Kenya National Ethical Review Committee

C/o Kenya Medical Research Institute

P.O. Box 54840, Nairobi, Kenya

Tel. 254-20-2722541

**IF THERE IS ANY PORTION OF THIS CONSENT AGREEMENT THAT YOU DO NOT UNDERSTAND, PLEASE TALK TO SOMEONE ON THE STUDY TEAM BEFORE SIGNING.**

You will receive a copy of the consent form to take home with you.

Subject's Name: _____

If Subject a minor

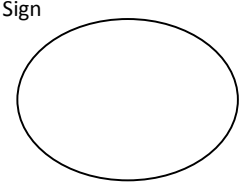
Parent/Guardian's Name: _____

Subject's or Parent/Guardian's Signature: _____ Date: _____

Permanent Address: _____

**If Adult Subject or Parent/Guardian Illiterate**

Thumbprint of Volunteer or Volunteer's Parent /Guardian if Unable to Sign



Witness's Name: _____

Witness's Signature: _____ Date: _____

Study Number: _____

**INDIVIDUAL OBTAINING CONSENT:** I certify that I have explained to the above individual the nature and purpose of this study, potential benefits, and possible risks associated with participation in this study. I have answered any questions that have been raised.

Name:_____

Signature: _____ Date: _____

# Appendix 2: Ethical clearance


KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org


**KEMRI/RES/7/3/1** **August 20, 2013**

**TO:** **JOHN WAITUMBI**
**PRINCIPAL INVESTIGATOR**

**THRO':** **DR. JUMA RASHID,**
**THE DIRECTOR, CCR,**
**NAIROBI**

27/8/2013

Dear Sir,

**RE:** **SSC NO. 1282 (*REQUEST FOR ANNUAL RENEWAL*): ACUTE FEBRILE ILLNESS SURVEILLANCE IN KENYA**

Thank you for the continuing review report for the period **11th July 2012** to **12th July 2013.**

This is to inform that during the 218th meeting of the KEMRI/ERC meeting held on 20th of August 2013, the Committee **conducted the annual review and approved** the above referenced application for another year.

This approval is valid from today **20th August 2013** through to **August 19, 2014**. Please note that authorization to conduct this study will automatically expire on**, August 19, 2014**. If you plan to continue with data collection or analysis beyond this date please submit an application for continuing approval to the **ERC** secretariat by **July 8, 2014.**

You are required to submit any amendments to this protocol and other information pertinent to human participation in this study to the SSC and ERC for review prior to initiation.

Yours faithfully,

KAB

**DR. ELIZABETH BUKUSI,**
**ACTING SECRETARY,**
**KEMRI/ETHICS REVIEW COMMITTEE**

## Appendix 3: Comparison of EBV prevalence between regions by Chi-square analysis

| | $X^2$-value | Degrees of freedom (df) | *p*-value |
|---|---|---|---|
| Lake Victoria region versus Kisii highland | 24.840 | 1 | < 0.0001 |
| Lake Victoria region versus semi-arid areas | 35.150 | 1 | < 0.0001 |
| Lake Victoria region versus coastal region | 2.919 | 1 | 0.0875 |
| Coastal region versus Kisii highland | 2.173 | 1 | 0.1405 |
| Coastal region versus semi-arid areas | 2.660 | 1 | 0.0557 |
| Kisii highland versus semi-arid areas | 0.328 | 1 | 0.5669 |

## Appendix 4: Comparison of AFI cases with viraemia ≥ 2000 copies/mL between regions

| | $X^2$-value | Degrees of freedom (df) | *p*-value |
|---|---|---|---|
| Lake Victoria region versus Kisii highland | 6.006 | 1 | 0.0143 |
| Lake Victoria region versus semi-arid areas | 7.094 | 1 | 0.0077 |
| Lake Victoria region versus coastal region | 1.461 | 1 | 02268 |
| Coastal region versus Kisii highland | 6.068 | 1 | 0.0138 |
| Coastal region versus semi-arid areas | 6.501 | 1 | 0.0108 |
| Kisii highland versus semi-arid areas | 0.0076 | 1 | 0.9304 |