PREVALENCE OF PLASMODIUM SPECIES AND CO-INFECTION WITH EPSTEIN-BARR VIRUS AMONG SELECTED PATIENTS ATTENDING ABUTH, ZARIA-NIGERIA

BY

SATI JOSEPH MOMOH

DEPARTMENT OF MICROBIOLOGY,

FACULTY OF LIFE SCIENCES,

AHMADU BELLO UNIVERSITY,

ZARIA

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Sati Joseph MOMOH, BSc (ABU ZARIA) 2012

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DEPARTMENT OF MICROBIOLOGY,

FACULTY OF LIFE SCIENCES,

AHMADU BELLO UNIVERSITY,

ZARIA

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DECLARATION

I declare that the work in this dissertation entitled:"**Prevalence of *Plasmodium* speciesand Co-infection with Epstein-Barr virus among selected Patients attending ABUTH, Zaria-Nigeria"**was carried out by me in the Department of Microbiology, Faculty of Life Sciences, Ahmadu Bello University, Zaria. The information derived from literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other institution.

_____	_____	_____
Name of Student	Signature	Date

CERTIFICATION

This dissertation entitled: "Prevalence**of *Plasmodium* species and Co-infection with Epstein-Barr virus among selected Patients attending ABUTH, Zaria-Nigeria"**by Sati Joseph MOMOH, meets the regulations governing the award of the degree of Master of Science in Microbiology of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

Dr. E.E. Ella, _____ _____
Chairman, Supervisory Committee,Signature Date
Department of Microbiology,

Faculty of Life Sciences, ABU Zaria.

Dr. (Mrs.) M. Aminu-Mukhtar, _____ _____

Member, Supervisory Committee,Signature Date

Department of Microbiology,

Faculty of Life Sciences, ABU Zaria.

Prof. I. O. Abdullahi,_____ _____
Head,Signature Date
Department of Microbiology,
Faculty of Life Sciences, ABU Zaria.

Prof. Kabir Bala,_____ _____
Dean, School of Postgraduate Studies, Signature Date ABU Zaria.

DEDICATION

This dissertation is dedicated first, to God- the reason I still breathe, to my Parents: Mr. Momoh Joseph Beida and Mrs. Momoh Justina;to the mother of my unborn children-Adora and to the best friend I have ever had- Late Mr. Gideon Gowok, who died of sickle cell disease. Do rest in peace, brother.

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ABSTRACT

Coinfection between *Plasmodium* species and Epstein-Barr virus (EBV) has been linked to many EBV-related cancers, especially in regions where malaria is endemic. The aim of this study was to determine the prevalence of *Plasmodium*speciesand co-infection with EBV among selected patients attending Ahmadu Bello University Teaching Hospital, Zaria-Nigeria. While microscopy was used in the detection and quantification of *Plasmodium* species, the enzyme-linked immunosorbent assay was used for the detection of IgG antibodies to EBVNA-1 and quantification of the selected patients' immune status ratio (ISR) to EBVNA-1. A total of 279 patients were enrolled in this study, out of which 44.8% (125/279) were male and 55.2% (154/279) were female. Based on their clinical presentations, the selected patients, were separated into three study groups-Febrile (58.8%:164/279), Cancer(27.2%:76/279) and the Sickle-cell group(14.0%:39/279). The only *Plasmodium* species found among the study population was *P. falciparum* with a prevalence of 63.4%. The highest prevalence of *falciparum* malaria was found among the cancer group(89.5%: 68/76) while the least was found among the febrile group(48.2%: 79/164). The female patients had higher *falciparum* parasitaemia (67.5%: 104/154) compared to their male counterparts (58.4%: 73/125). Malaria infection was highest among the 61-70age-group (100%; 4/4) and least was among the 21-30 age-group (52.8%; 28/53) ($P<0.05$). Also, a higher parasitaemia was found among the divorced (83.3%: 5/6) compared to the married (54.6%: 89/163) ($P>0.05$). Those who had tertiary education had lower*falciparum* malaria (41.2%: 39/94) compared to those who have had only primary education (87.5%: 56/64)($P<0.05$). All the patients had IgG antibodies to EBVNA-1. However, the highest ISRwas found among cancer patientswho also had malaria(1.34; 68/76) while the least ISR was found among malaria-negative febrile patients (0.59; 85/164) ($P<0.05$; OR=7.08). Sixty-three point four percent (63.4%) of the study population were co-infected with EBV and *P. falciparum*. Thefactorsfound in this study to predispose to EBV-related cancers wereexposure to X-rays (59.86%: 167/279)($P<0.05$) and a family history of cancer (15.4%; 43/279)($P<0.05$). There was no significant statistical association between genotype and blood group of study patientsand infection with*P. falciparum* malaria ($P>0.05$). Malaria is still endemic in Zariaand *P. falciparum*is the most prevalent species (63.4%). More research still needs to be done in the ongoing fight to roll back malaria, especially as*falciparum*malaria in co-infection with EBV has been shown to increase ISR levels, a risk factor for EBV-related cancers.

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CHAPTER ONE

1.0 INTRODUCTION

Infectious agents are important contributors to the global cancer burden particularly in Africa. Of the 12.7 million new cancer cases diagnosed worldwide in 2008, about two million were attributable to infectious agents, of which 1.6 million occurred in less developed regions of the world (Odutola, *et al.*, 2016). In its 2009 review of the carcinogenicity of infectious agents, the International Agency for Research on Cancer (IARC) found sufficient evidence to conclude that seven viruses: human papillomavirus (HPV), Epstein–Barr virus (EBV), hepatitis B virus (HBV), hepatitis C virus (HCV), human immunodeficiency virus type-1 (HIV-1), Kaposi's sarcoma herpes virus (KSHV), and human T-cell leukemia virus type-1 (HTLV-1), were carcinogenic to humans (Odutola, *et al.*, 2016).

In March, 1964 the Lancet Medical Journal published a remarkable piece of research from three scientists called Anthony Epstein, Yvonne Barr, and Burt Achong. They had discovered the first human virus that is the etiology of cancer, and the virus was named after them: Epstein-Barr virus, EBV (Smith, 2014). Owing to Burkitt's intrepid studies in Africa and Epstein's persistence, we now know that EBV infection plays a role in diverse types of cancer, including lymphomas, nasopharyngeal cancers and some stomach cancers (Smith, 2014).

Epstein-Barr virus was the first human virus to be directly implicated in carcinogenesis (Thompson and Kurzrock, 2004). It infects >90% of the world's population (Thompson and Kurzrock, 2004). Although most humans coexist with the virus without serious sequelae, a small proportion progress to develop tumors (Thompson and Kurzrock, 2004). Normal host populations can have vastly different susceptibility to EBV-related tumors as demonstrated by geographical and immunological variations in the prevalence of these cancers (Thompson and

Kurzrock, 2004). The presence of this virus has also been associated with epithelial malignancies arising in the gastric region and the breast, although some of this work remains in dispute(Thompson and Kurzrock, 2004). Epstein-Barr virus uses its viral proteins, the actions of which mimic several growth factors, transcription factors, and anti- apoptotic factors, to usurp control of the cellular pathways that regulate diverse homeostatic cellular functions (Thompson and Kurzrock, 2004).

The Epstein-Barr virus (EBV) is classically associated with malignancies such as Burkitt's lymphoma, B-cell lymphoproliferative syndromes, nasopharyngeal carcinoma, Hodgkin's disease, T-cell lymphomas, and gastric carcinoma, as well as being the causal agent for infectious mononucleosis (Pagano, 1999). The relation of the virus to the malignancies varies from primary etiologic agent to necessary or contributory cofactor (Pagano, 1999). Most of the malignancies occur after years of viral dormancy and are accompanied or triggered by viral reactivation, in contrast to infectious mononucleosis, which results from primary infection with EBV (Pagano, 1999).

The Epstein–Barr virus also called human herpesvirus 4 (HHV-4), is one of eight viruses in the herpesviridae family, and is one of the most common viruses in humans. It is best known as the cause of infectious mononucleosis (glandular fever)(Cherry-Peppers *et al.,* 2003; Maeda *et al.*, 2009). It is also associated with particular forms of cancer, such as Hodgkin's lymphoma and conditions associated with human immunodeficiency virus (HIV), such as hairy leukoplakia and central nervous systemlymphomas (Cherry-Peppers *et al.,* 2003; Maeda *et al.,* 2009).

The EBV is approximately 122 nmto 180 nm in diameter and is composed of a double helix DNA of about 172,000 base pairs codingfor about 85 genes (Amon, 2004). The DNA is surrounded by a protein nucleocapsid. This nucleocapsid is surrounded by a tegument made of protein, which in turn is surrounded by an envelope containing both lipids and surface projections of glycoproteins which are essential forinfection of the host cell (Odumade and Hogquist, 2011).

Burkitt's lymphoma, a childhood cancer common in parts of sub-Saharan Africa, has been associated with EBV and malaria (Mutalima *et al.*, 2008). A protein produced by the malaria parasite can trigger an aggressive and torturous form of cancer called Burkitt's lymphoma(Khamsi, 2007).It was found that the cysteine-rich inter-domain region 1α (CIDR1α) of the*Plasmodium falciparum* membrane protein 1,is a polyclonal B cell activator that preferentially activates the memory compartment where EBV is known to persist (Chêne *et al.,* 2007). It has been shown that CIDR1α binds to the EBV-positive B cell line and increases the number of cells switching to the viral lytic cycle as measured by green fluorescent protein (GFP) expression driven by a lytic promoter (Chêne *et al.,* 2007). The virus replication in CIDR1α-exposed cultures was directly proportional to the number of GFP-positive cells (lytic EBV) and to the increased expression of the EBV lytic promoter BZLF1 (Chêne *et al.,* 2007). Furthermore, CIDR1α stimulated the replication of EBV in peripheral blood mononuclear cells derived from healthy donors and children with Burkitt's lymphoma (Chêne *et al.,* 2007). Also,*P. falciparum* antigens such as CIDR1α can directly induce EBV reactivation during malaria infection, increasing the risk of Burkitt's lymphoma development for children living in malaria-endemic areas (Chêne *et al.,* 2007).The Burkitt's lymphoma illness causes horrendous suffering. The immune cells turn cancerous and tumours develop in the lymph nodes, often making the tongue

swell and cheeks bulge (Khamsi, 2007). The tumour is characterized by chromosome translocations (Manolov and Manolova, 1972). Usually, the terminal portion of chromosome 8 containing the c-*myc* gene is translocated to chromosome 14 (Taub, 1982), near the enhancer of the immunoglobulin heavy-chain locus (Hayday, 1984).

It has been discovered that attack by*Plasmodium falciparum* malaria results in alteration of the T-cell subpopulations so that, *in vitro*, B lymphocytes infected with EBV proliferate abnormally to secrete large amounts of immunoglobulin and antibody. This phenomenon offers some explanation for the increased incidence of Burkitt's tumor and the high levels of immunoglobulin found in people living in areas where *P. falciparum* malaria is common (Greenwood, 1974).

Malaria is caused by parasites that are spread by mosquitoes. The Anopheles mosquitoes transmit the malaria parasites that cause malaria in humans(Jimoh *et al.,* 2007). In adults, its common symptoms are headache, weakness, fever, aches and pains, high body temperatures and bitterness of the mouth (loss of appetite) while in children, in addition to the already mentioned symptoms, it may also manifest in more than normal sleeping, nausea and vomiting(Jimoh *et al.,* 2007). It is a serious disease affecting children and adults but its consequences are graver among children and pregnant women (Jimoh *et al.,* 2007).

Malaria is endemic in Nigeria and is a leading cause of morbidity and mortality in the country. The disease accounts for 25% infant mortality and 30% of childhood mortality in Nigeria alone(Jimoh *et al.,* 2007). Nigeria can be divided into three major malaria epidemiological zones, namely, forest, savannah and the grass-land zones(Jimoh *et al.,* 2007). The forest zone consists of coastal areas stretching from Lagos in the South-Western Nigeria to the forest areas in the Eastern Nigeria, up to the Northern portion of the forest zone of Oyo state. The Savannah zone

consist of areas north of Oyo state to the central areas of Kogi and Benue states, while the Grass-land zone consists of the Nothern parts of Nigeria (Jimoh *et al.,* 2007).

The most dominanat species of Anopheles mosquito in Nigeria are *Anopheles funestus, A. gambiae, A. complex, A. arabiensis, and A. melas*. The dominant vector in the forest zone is the *A. melas* while the dominant vectors in the Savannah zone are a combination of *A. melas* and *A. arabiensis* (Jimoh *et al.,* 2007). The dominant vector in the Grass-land zone is *A. arabiensis.* The most deadly strain of malaria, *Plasmodium falciparum* has been reported in all zones and other parts of Africa (Jimoh *et al.,* 2007; WHO, 2016).

It is widely accepted that the sickle cell trait protects the carrier from malaria because only a small number of *P. falciparum* are able to infect sickled cells due to their shapes and increased rates of phagocytosis of infected sickled cells (Lamonte *et al.,* 2012). It can therefore be implied that an inverse association of Burkitt's lymphoma with the sickle-cell trait (AS haemoglobin) would provide strong evidence for the role of intense falciparum malariain the increased incidence of EBV-related tumors (Morrow, 1985).

1.1 Statement of the Research Problem

Epstein-Barr virus, a ubiquitous B-lymphotrophic herpesvirus (Hsu and Glaser, 2000) is an accepted carcinogen, since experimental studies have clearly established its presence in tumour cells of several malignancies (Gulfaraz and Muhammad, 2014). Some of these malignancies include Burkitt's lymphoma, lymphomas associated with immunosuppression, Hodgkin's disease, other non-Hodgkin's lymphomas, nasopharyngeal carcinoma, gastric adenocarcinoma, lymphoepithelioma-like carcinomas and immunodeficiency-related leiomyosarcoma (Hsu and Glaser, 2000).Cancer is one of the leading causes of death worldwide and research focused on

understanding itsaetiology and pathogenesis is a major challenge. Over a period of 20 years, global mortality from cancer has increased from 5.779 million in 1990 to 7.978 million in 2010. This represents an increase of approximately 2% per year (Gulfaraz and Muhammad, 2014). It has also been estimated that oncogenic viruses play an etiological role in the development of 12% of all human malignancies (Gulfaraz and Muhammad, 2014).

Epstein-Barr virus has continued to attract considerable attention, primarily due to its oncogenic properties and its association with a number of malignancies including post-transplant lymphoproliferative disease (PTLD) (Gulfaraz and Muhammad, 2014).About 80% of PTLD cases have been attributed to EBV (Gulfaraz and Muhammad, 2014).Some published researches have estimated that approximately 1.5% of transplant recipients develop PTLD while 50% of these die within the first year (Gulfaraz and Muhammad, 2014). It has been estimated that the risk of developing PTLD and dying from it was the same for both sexes (Gulfaraz and Muhammad, 2014).For the age-group of 0-14 years, it was estimated that mortality of 90.5%, 33.3% and 15.2% were found in regions where Burkitt's lymphoma is endemic, intermediate or sporadic respectively (Gulfaraz and Muhammad, 2014). Burkitt's lymphoma is the fastest growing human tumour (Robbiani *et al.,* 2015). The annual incidence has been estimated at 40-50 per million children younger than 18 years (Robbiani *et al.,* 2015).A vast majority of the world's population exhibits antibodies to EBV and the infection usually occurs early in childhood (Thompson and Kurzrock, 2004).

Malaria is the number one killer of all parasitic diseases and more than 80% of the deaths worldwide occur in sub-Saharan Africa (Brooks *et al.,* 2010). According to the World Health Organization, in 2015, there were about 214 million cases of malaria. The disease caused around 438,000 deaths of which 306,000 were of children under 5 years (WHO, 2015)and about 3.2

billion people, almost half of the world's population are at risk of malaria (WHO, 2016). The Centre for Disease Control further reported malaria to be the number one cause of death in Nigeria (CDC Nigeria, 2015). The majority of these deaths are due to *Plasmodium falciparum* malaria (Targett and Greenwood, 2008).Understandably, the focus of enhanced research and malaria control has been primarily on *Plasmodium falciparum* malaria, given the mortality and severity of disease associated with this species (Targett and Greenwood, 2008).

A co-infection with *Plasmodiumfalciparum*malaria and EBV is a major risk factor for endemic Burkitt lymphoma (eBL), one of the most prevalent paediatric cancers in Equatorial Africa (Chattopadhyay*et al.*, 2012). A coinfection with malaria parasite and EBV can increase the mortality of malaria patients. Children in sub-Saharan Africa become infected with EBV in infancy and it is quite difficult to distinguish from other mild childhood illnesses. Almost all African children are infected with EBV by the age of 6-12 months(FNIL, 2016). Since both of these infections occur around the same time in these children, many are at high risk for co-infection before the age of 1 (FNIL, 2016).

Finally, the chance of any individual developing an EBV-related lymphoma is modulated by ethnic, geographic (areas with holoendemic malaria), genetic, immunologic and other infectious cofactors (Kanakry and Ambinder, 2014).Furthermore, individuals said to carry the sickle cell trait, were in fact highly protected against malaria, thus explaining the high prevalence of this mutation in geographical areas where malaria is endemic (Instituto Gulbenkian de Ciencia, 2011). These findings lead to the widespread belief in the medical community that understanding the mechanism whereby sickle cell trait protects against malaria would provide critical insight into developing treatment or a possible cure for this devastating disease, responsible for over a million premature deaths in sub-Saharan Africa (Instituto Gulbenkian de Ciencia, 2011).

1.2 Justification of the study

This study will determine the prevalence of*Plasmodium* species, as well as the parasite density in the study area. Current information on the prevalence of EBV and the ISR of patientsto EBVNA-1in the study area will be provided. Since EBVNA-1 is an established tumor marker (a substance that is produced by cancer or by other cells of the body in response to cancer or certain benign conditions) the presence of measureable antibodies to it can provide data on possible risk for carcinogenesis. A corresponding data on the complications associated with a co-infection of EBV and *Plasmodium falciparum,*especially that predisposing to cancer will be brought to fore. If other *Plasmodium* species aside*P. falciparum*are present in the study area, an association between them and EBV will be determined with the hope of providing current information. Furthermore, the study will determine the protective role of the sickle-cell trait against malaria which has been a subject of much speculation and debate among researchers. More so, the likely risk factors associated with EBV-related cancers as well as EBV and malariaco-infection will be determined among patients in the study area. Finally data generated from this research will be useful in both preventive and control measures as well as in the treatment of EBV, malaria and EBV-related cancers.

1.3 Aim of the Study

The aim ofthe study was to determine the prevalence of *Plasmodium*species and co-infection with Epstein-Barr virusamong selected patients attending Ahmadu Bello University Teaching Hospital, Shika-Zaria, Nigeria.

1.4 Objectives of the Study

The objectives of the study were to:

1. Detect the presence of *Plasmodium* species and their density among the selected patients using light microscope and the atlas of Medical Parasitology.
2. Screenfor anti-EBVNA-1 (IgG) and measure the Immune Status Ratio (ISR) of the selectedpatientsto EBVNA-1usingELISA.
3. Determine co-infection of Epstein-Barr virus and *Plasmodium* species among the selected patients.
4. Determine likely risk factors for EBV-related cancersand co-infection with EBVand *Plasmodium* species.

CHAPTER TWO

2.0 LITERATURE REVIEW

2. 1. Malaria

Malaria is the number one killer of all parasitic diseases and more than 80% of the deaths worldwide occur in sub-Saharan Africa (Brooks *et al.*, 2010). More than a century after the discovery of *Plasmodium* spp. parasites, the pathogenesis of severe malaria is still not well understood (Wassmer *et al.*, 2015). The majority of malaria cases are caused by *Plasmodium falciparum* and *Plasmodium vivax*, which differ in virulence, red blood cell tropism, cyto-adhesion of infected erythrocytes, and dormant liver hypnozoite stages (Wassmer *et al.*, 2015). The much quoted figures for malaria deaths and clinical cases (around one million deaths and 300-500 million clinical cases per annum) are still the best estimates available (Targett and Greenwood, 2008). The majority of these deaths are due to *Plasmodium falciparum* malaria (Targett and Greenwood, 2008).

Malaria is a major global infectious disease caused by parasitic protozoans of the genus *Plasmodium*. Of the five *Plasmodium* species that infect humans, *Plasmodium falciparum* and *Plasmodium vivax* cause the majority of cases, and *P. falciparum* is the most virulent and responsible for the majority of deaths (WHO, 2013). Despite recent reductions in the overall malaria case incidence, malaria remains a leading cause of morbidity and mortality in the developing world (WHO, 2013). In 2012, there were an estimated 207 million cases of malaria and over 600,000 deaths (WHO, 2013).

The majority of malaria deaths (90%) occur in children in Africa, where *falciparum* malaria accounts for as many as one in six childhood deaths and is the biggest killer of African children between the ages of 1 and 4 years (Murray *et al.*, 2012). Malaria affects 3.3 billion people, or half of the world's population. WHO estimates 216 million cases of malaria occurred in 2010,

with 81% in the African region. WHO also estimates there were 655,000 malaria deaths in 2010, with 91% in the African Region, and 86% were children under 5 years of age (United States Embassy in Nigeria, 2011). Malaria is the 3rd leading cause of death for children under five years worldwide, after pneumonia and diarrheal disease (United States Embassy in Nigeria, 2011).

Outside Africa, there are a variety of transmission settings where *P. falciparum*, *P. vivax*, or both are present. In lower transmission settings in South America, India, and Southeast Asia, adult populations are at higher risk for severe malaria (Miller *et al.*, 2002). Malaria is a complex disease, and the spectrum of disease manifestations differs between children and adults (Miller *et al.*, 2002). Only a small minority of infections, less than 1–2%, leads to severe malaria (Marsh, 1992). Symptoms can range from none, in individuals with asymptomatic parasitaemia, to mild, in patients with undifferentiated fever, to severe, in patients with life-threatening anemia, metabolic acidosis, cerebral malaria (CM), and multiorgan system involvement (Beales *et al.*, 2000).

Malaria is an acute febrile illness. In a non-immune individual, symptoms appear 7 days or more (usually 10–15 days) after the infective mosquito bite (WHO, 2015). The first symptoms – fever, headache, chills and vomiting – may be mild and difficult to recognize as malaria. If not treated within 24 hours, *P. falciparum* malaria can progress to severe illness, often leading to death (WHO, 2015). Children with severe malaria frequently develop one or more of the following symptoms: severe anaemia, respiratory distress in relation to metabolic acidosis, or cerebral malaria. In adults, multi-organ involvement is also frequent. In malaria endemic areas, people may develop partial immunity, allowing asymptomatic infections to occur (WHO, 2015).

Due to the breakdown of malaria control programs, the constant emergence of drug resistant parasites, and possibly, climatic changes, malaria poses a major problem for the developing

countries (Weatherall*et al.,* 2002). In addition, because of the speed of international travel, it is being seen with increasing frequency that malaria is an imported disease in non-tropical countries (Weatherall*et al.,* 2002). The burden caused specifically by antimalarial drug resistance is more difficult to quantify (Philips and Philips-Howard, 1996). Understandably, the focus of enhanced research and malaria control has been primarily on *Plasmodium falciparum* malaria, given the mortality and severity of disease associated with this species (Targett and Greenwood, 2008). There is no single factor that confers the greatest degree of influence on the spread of drug resistance, but a number of plausible causes associated with an increase have been acknowledged. These include aspects of economics, human behavior, pharmokinetics, and the biology of vectors and parasites (Olashehinde *et al.,* 2015).

Patients' adherence to malaria treatment is an important factor in determining the therapeutic response to antimalarial drugs as it contributes to the patients' complete recovery and prevents the emergence of parasite resistance to antimalarial drugs (Pereira *et al.,* 2011). Hence, the full benefit of many effective medications will be achieved only if patients adhere to prescribed treatment regimens (LarsOstenberg and Terrence Blaschke, 2005). The implication, then, is that taking an ineffective drug can result in severe or protracted disease and even death (Goodman and Mills, 2000).

Historically, *P. vivax* has been considered a relatively benign parasite, but recently there has been a renewed appreciation that it carries a significant morbidity and mortality burden in endemic regions (Dondorp *et al.,* 2005). Furthermore, a 5–15% mortality rate was reported in the early neurosyphilis therapies of patients with *P. vivax* (Dondorp *et al.,* 2005). Part of the explanation for the "benign" reputation, despite the evidence for mortality, is that vivax parasites are highly restricted to reticulocytes and therefore cannot achieve the same high parasite biomass as *P. falciparum* (Miller *et al.,* 2002). A second difference is that *P. vivax* possesses relatively

poor infected erythrocytes (IE) adhesive capacity compared with *P. falciparum* (Dondorp *et al.*, 2010).

People living in malaria endemic areas are often exposed to several pathogens simultaneously. Malaria may be acquired together with a bacterial, viral parasitic or other malarial species infection. These co-infections could arise due to chance because of host susceptibility and co-circulation of various disease agents. Alternatively, malaria may increase the risk for another infection or vice versa. Overlapping clinical features in these co-infections may pose serious diagnostic challenges, especially in impoverished areas where the capacity for laboratory testing is limited. Simultaneous illnesses may be more severe or produce additional sequelae compared to a single infection (Deen, 2014).

2.1.1. The Plasmodium Life Cycle and Pathogenesis

After a mosquito bite, sporozoites travel to the liver to infect hepatocytes and develop into approximately 30,000 merozoites that are released into the bloodstream (Gazzinelli *et al.*, 2014). Repeated cycles of red blood cell (RBC) invasion, replication and merozoite release will result in the exponential growth of the parasite population and lead to disease. *Plasmodium vivax* sporozoites also differentiate into dormant hypnozoites that may cause disease relapses. Infected RBCs will circulate containing ring-stage parasites, and a small proportion of merozoites will develop into male and female gametocytes that infect mosquitoes, completing the parasite life cycle (Gazzinelli *et al.,* 2014). The removal of infected RBCs by splenic macrophages or the uptake of free haemozoin results in the activation of innate immune receptors and cytokine storm. The circulating cytokines will cause paroxysms and induce the expression of adhesion molecules by endothelial cells, which mediate parasite sequestration(Gazzinelli *et al.,* 2014).

The sequestration of infected RBCs disrupts blood flow, promotes blood clots, injures endothelial cells and ruptures vascular walls, leading to the extravasation of vascular content and local tissue inflammation (Gazzinelli *et al.,* 2014). These mechanisms contribute to acute respiratory distress, cerebral malaria or placental malaria. The sequestration of infected reticulocytes is less intense and these syndromes are not common in *P. vivax* malaria (Gazzinelli *et al.,* 2014). Haemolysis of infected and uninfected RBCs, uptake of altered RBCs by splenic macrophages and cytokine-induced impairment of erythropoiesis causes anaemia. Free haemoglobin catalyses oxidative damage, hypoxia and lactic acidosis, promoting metabolic acidosis, which is aggravated by the altered renal function that is observed in patients with malaria (Gazzinelli *et al.,* 2014).

Plasmodium falciparum and *P. vivax* within RBC develop over 48 hours, producing around 20 merozoites in a mature parasite, each able to invade other RBC. A small proportion of asexual parasites converts to gametocytes that are critical for the transmission of the infection to others through female anopheline mosquitoes but cause no disease (Weatherall*et al.,* 2002). Here the strategy of *P. vivax* differs from that of *P. falciparum*. *Plasmodium vivax* develops into gametocytes soon after release of merozoites from the liver; *P. falciparum* gametocytes develop much later. WHO recommends that all cases of suspected malaria be confirmed using parasite-based diagnostic testing (either microscopy or rapid diagnostic test) before administering treatment. Early diagnosis and treatment of malaria reduces disease and prevents deaths. It also contributes to reducing malaria transmission. The best available treatment, particularly for *P. falciparum* malaria, is artemisinin-based combination therapy (ACT) (WHO, 2015).

2.1.2. Invasion of Red Blood Cells (RBCs)

The sequence of invasion is probably similar for all *Plasmodium* spp. The parasite must engage receptors(Chitnis, 2001) on RBC for binding and undergoing apical reorientation junction formation and signaling (Drovak *et al.*, 1975; Aikawa *et al.*, 1978). The parasite then induces a vacuole derived from the RBC plasma membrane and enters the vacuole by a moving junction (Adams, 1990). Three organelles on the invasive (apical) end of parasites (rhoptries, micronemes, and dense granules) define the phylum Apicomplexa. Receptors for invasion of RBC by merozoites and for invasion of liver by sporozoites are found in micronemes on the cell surface, and in rhoptries (Adams, 1990; Weatherall*et al.*, 2002).

The distribution of these receptors within an organelle may protect the parasite from antibody-mediated neutralization, as the release after contact with the RBC may limit their exposure to antibody. A critical question in parasite biology remains the identification of the signaling pathways to release organellar contents on contact with a host RBC (Weatherall*et al.*, 2002). Malaria parasites have intracellular signaling pathways through phosphoinositide, cyclic AMP (cAMP), and calcium-dependent pathways (Kappe, 1999). What remains completely unknown is which merozoite surface molecules recognize the RBC surface and signal for the invasion process (Kappe, 1999). These events include release of critical molecules from apical organelles and the initiation of the actin-myosin moving junction that brings the parasite within the vacuole that forms in the RBC. The TRAP protein interacts with skeletal proteins in malaria sporozoites and in *Toxoplasma gondii* (Kappe, 1999) but the equivalent molecule for merozoites has yet to be identified (Weatherall*et al.*, 2002).

Both *P. falciparum* and *P. vivax* can cause severe anemia, but only *P. falciparum* causes the multiple complications of cerebral malaria, hypoglycemia, metabolic acidosis, and respiratory distress. Certain differences in the biology of the two parasites partially explain the differences in

patterns of disease. First, *P. falciparum* can invade a large percentage of the RBC, whereas *P. vivax* is limited to reticulocytes. Similar differences are found between virulent and avirulent *P. yoelii*. Both invade reticulocytes preferentially, but once the reticulocytes are consumed, virulent *P. yoelii* can invade all RBC, leading to higher parasitemia and death. A study of severe compared to uncomplicated falciparum malaria suggested a similar pattern, with the virulent *P. falciparum* invading all RBC and the avirulent parasites invading only a subpopulation (Chotivanich, 2000).

A second difference is the surprising redundancy of invasion pathways in *P. falciparum*, lacking in *P. vivax*. *P. vivax* invades only Duffy blood group-positive RBC and is largely limited to reticulocytes (Miller *et al.,* 1976). In West Africa, where the Duffy blood group is missing on RBC, *P. vivax* essentially disappeared. The Duffy negative blood group has also occurred independently in Papua New Guinea an area of high endemicity of *P. vivax* (Zimmerman, 1999). The limitations in invasion of *P. vivax* have led to the discovery of two families of parasite receptors:

i) the parasite molecule that binds to the Duffy blood group system and Duffy binding-like (DBL) homologous proteins of *P. falciparum* and *P. knowlesi* (Adams, 1992) and

ii) the parasite reticulocyte-binding proteins of *P. vivax*(Galinski *et al.,* 1992) and reticulocyte binding-like (RBL) homologous proteins of *P. falciparum*(Rayner *et al.,* 2001) and *P. yoelii* (Keen *et al.,* 1994).

The various members of the DBL and RBL families may recognize different RBC receptors than the Duffy blood group or the receptor on reticulocytes. The receptor grouping into DBL and RBL refers to the family of homologous parasite proteins, not the specificity on the RBC for binding (Weatherall*et al.,* 2002).

2.1.3. Clinical Features of Malaria

The signs and symptoms of malaria infection in humans are caused by the asexual blood stage of the parasite (Weatherall *et al.,* 2002). The ratio of numbers of deaths to infections from malaria suggests that infection with blood stage parasites may result in a wide range of outcomes and pathologies (Weatherall *et al.*, 2002). Indeed, the spectrum of severity ranges from asymptomatic infection to rapidly progressive, fatal illness. The clinical presentation of malaria infection is particularly influenced by host age, by immune status with respect to malaria and pregnancy, and by the species, genotype, and perhaps the geographical origin of the parasite (Weatherall *et al.*, 2002).The characteristics of *falciparum* malaria have been most extensively studied in African children (Weatherall *et al.,* 2002). Symptoms may appear on average 12 days (but occasionally 6 months or more) after inoculation of sporozoites into the bloodstream. An infection may be asymptomatic in those with acquired or innate immunity to malaria; others with no or partial immunity may suffer from a severe acute illness (Weatherall *et al.,* 2002;WHO, 2015).

(a) Uncomplicated or Mild Malaria: Prodromal symptoms of malaria include headache (which is often severe and dominates the presenting complaint), myalgia, and coughing that preceDe The typical sequence of shaking chills, fever, and sweating associated with a paroxysm of fever (Weatherall *et al.,* 2002). The erythrocytic cycle in falciparum malaria is usually synchronized so, in the initial stages of infection, fever occurs on days 1 and 3 (and thus is a tertian fever). In advanced infections the pattern of fever becomes less regular, even continuous (Weatherall *et al.*, 2002). Nausea, vomiting, diarrhea, and abdominal pain may accompany fever. In an uncomplicated infection, signs are few, with the notable absence of lymphadenopathy or rash, but include splenomegaly and mild jaundice (Weatherall *et al.*, 2002). If the course of treatment is incomplete or if the parasites are resistant to the treatment given, then parasites may

recrudesce and once more cause a patent infection. Follow-up of treated cases is therefore essential (Weatherall *et al.,* 2002;WHO, 2015).

(b) Severe Malaria: In some children, malaria causes more serious disorders. They may present with prostration or inability to take oral fluids or, in younger children, inability to suckle. As disease progresses they may exhibit a number of syndrome(s) of severe disease including coma, respiratory distress, anemia, and hypoglycemia and may also have a high rate of bacteremia (Snow *et al.,* 1999). On admission, children usually give a history of only a few days' illness. Exclusion of intercurrent diseases may be difficult. The parasitaemia is an unreliable guide to the disease severity, and severe disease may occur in the face of undetectable parasitemia where the parasites are sequestered and their growth cycle is tightly synchronized (Weatherall *et al.,* 2002). Thus, clinical assessment must always seek to exclude other illnesses, in particular acute respiratory infection, bacterial meningitis, encephalitis, Reye's syndrome and septicemia which may mimic respiratory distress, coma, and/or multisystem disease due to malaria (Weatherall *et al.,* 2002; WHO, 2015).

(c) Cerebral Malaria: The cardinal signs of 'cerebral malaria' are reduced consciousness and coma. A core of patients have signs consistent with a diffuse metabolic encephalopathy, although patients who present with reduced consciousness may have other distinct pathophysiological disturbances including seizures, metabolic acidosis, and hypoglycemia. The pathology of 'cerebral malaria' is linked with the sequestration of parasitized erythrocytes in postcapillary venules of the cerebral circulation, although the functional disturbance(s) that lead to an encephalopathy are poorly understood (Taylor *et al.,* 1993).

The depth of coma can be measured at the bedside using a simplification of the Glasgow coma scale devised initially in Blantyre, Malawi (English, 1996). As unconsciousness deepens, the

patient fails to localize pain and may demonstrate abnormal posturing (decorticate rigidity, decerebrate rigidity, and opisthotonus), pupillary changes, absent corneal reflexes, and abnormal respiratory patterns including hypoventilation and periodic breathing (English, 1997). Retinal hemorrhages are common (Weatherall *et al.*, 2002).

Seizures are a prominent feature of cerebral malaria. Children may suffer simple febrile seizures, have prolonged or multiple seizures, or have features suggesting a focus of epileptic activity, including localized motor seizures. Status epilepticus and prolonged post-ictal periods are not uncommon (Weatherall *et al.*, 2002). Interestingly, simultaneous EEGs and video recordings of comatose clinical patients have shown that generalized seizures may occur with minimal physical signs, such as twitching of finger(s), conjugate or nystagmoid deviation of the eyes, or hypoventilation with excessive salivation (Miller *et al.*, 1971). Such patients may recover consciousness after anticonvulsant therapy. Similarly, children presenting with metabolic acidosis and/or hypoglycemia may recover after appropriate treatment (Weatherall *et al.*, 2002).

The progression of signs in cerebral malaria is variable, but sometimes a cephalo-caudal progression of signs is seen including isolated cranial nerve palsies and brain stem signs culminating in respiratory arrest. This sequence of events and direct measurement of cerebrospinal fluid (CSF) pressure in children presenting in a coma has suggested that, unlike nonimmune adults with cerebral malaria, at least some children with cerebral malaria may have intracranial hypertension (Dondrop *et al.*, 2000). This has led to re-evaluation of the risk of early lumbar puncture in excluding meningitis in parasitemic, comatose children, and now some clinicians suggest delaying lumbar puncture and commencing empirical antibiotic treatment, to cover bacterial meningitis, with concurrent antimalarial treatment (English, 1996).

Children who recover from cerebral malaria usually do so within 48 hours, although a significant minority suffers from neurological sequelae(English, 1996). Up to 15% of children may have hemiplegia, ataxia, dysphasia, hearing difficulty, visual problems including cortical blindness, or epilepsy. Over half of these children make a full recovery, but there is a residue of serious disability caused by cerebral malaria, including abnormal cognitive function and behavior (English, 1996).

(d) Anemia: The blood stage of *falciparum* malaria may cause life-threatening anemia, and haemoglobin of less than 5 g/dL is considered to represent severe disease. Anemia may become worse after treatment begins, particularly if the parasitemia is high (Berkley *et al.,* 1999). The anemia is typically normocytic and normochromic, with a notable absence of reticulocytes, although microcytosis and hypochromia may be present due to the very high frequency of alpha and beta thalassemia traits and/or iron deficiency in many endemic areas (Berkley *et al.,* 1999). However, difficulties in assessing, not only the absolute, but also the 'functional' iron stores complicate the diagnosis and treatment of iron deficiency in the context of acute malaria infection (Weatherall *et al.,* 2002). In such circumstances the only diagnostic test of iron deficiency may be the response to iron supplementation. The indication(s) for and duration of treatment have not, however, been established. Although chronic haemolysis may increase folate requirements, a frank deficiency is uncommon in children presenting with acute malaria, at least in East Africa (Weatherall *et al.*, 2002; WHO, 2015).

The pathophysiology of severe anemia is a complex but relatively neglected area of study (Prada *et al.,* 1993). Certainly, malaria gives ample reasons for both increased destruction and reduced production of red cells. Red blood cells are destroyed as parasites complete their growth cycle, although some parasites may be removed from erythrocytes as immature ring forms by

phagocytic cells (Prada *et al.,* 1993). Infected erythrocytes may also be phagocytosed by macrophages following opsonization by immunoglobulins and/or complement components. Other effector cells and mechanisms are less well defined but may include antibody-dependent cytotoxicity and natural killer (NK) cells (Weatherall *et al.,* 2002).

The survival of uninfected erythrocytes is also reduced. The activity and the number of macrophages are increased in malarial infection. Moreover, the signals for recognition of uninfected erythrocytes for removal by macrophages are enhanced. Uninfected erythrocytes bind increased amounts of immunoglobulin and/or complement as detected in the direct antiglobulin test (DAT or Coomb's Test) (English *et al.,* 1996; Crawley, 1996). The specificity of the immunoglobulins on the surface of the red cells has remained controversial. These antibodies do not have a particular specificity but are more likely to represent immune complexes absorbed onto the surface of red blood cells by complement receptors including CR1 (CD35) (Mendis *et al.,* 2001).

No discussion of the pathology of malarial anemia is complete without consideration of 'blackwater fever.' The sudden appearance of haemoglobin in the urine indicating severe intravascular haemolysis leading to haemoglobinemia and haemoglobinuria received particular attention in early studies of anemia in expatriates living in endemic areas (Mota, 2001). There was an association between blackwater fever and the irregular use of quinine for chemoprophylaxis. This drug can act as a hapten and stimulate production of a drug-dependent complement-fixing antibody. Recent studies of sudden intravascular hemolysis have shown it is rare in Africa, but more common in Southeast Asia and Papua New Guinea, where it is associated with G6PD deficiency and treatment with a variety of drugs including quinine, mefloquine, and artesunate (Mota, 2001).

Reticulocytopenia has been observed in numerous clinical studies of malarial anemia. The histopathological study of the bone marrow of children with malarial anemia shows erythroid hyperplasia, with dyserythropoiesis, cytoplasmic and nuclear bridging, and irregular nuclear outline (Frevert, 1993). The concentrations of tumor necrosis factor α (TNF-α) and interferon (IFN)-γ have been correlated with the severity of the disease (Chitnis, 2001) and high levels of TNF-α have been shown to suppress erythropoiesis (Drovak *et al.,* 1975). These cytokines may also contribute to increased erythrophagocytosis. The possibility has been raised that high levels of the Th2-type cytokine interleukin-10 (IL-10) might prevent the development of severe malarial anemia. Low levels of IL-10 have been described in African children with severe malarial anemia (Drovak *et al.,* 1975). However, the mechanism of protection from anemia by IL-10 is unclear (Aikawa *et al.,* 1978). The hypothesis that parasite products directly stimulate the production of inflammatory cytokines, including TNF-α, has been widely promoted. The glycosylphosphatidylinositol (GPI) anchor of malarial membrane proteins may cause cellular dysfunction, but a role for this toxin in dyserythropoiesis remains to be established (Aikawa *et al.,* 1978).

Other toxic products may exist during its blood stage, the malaria parasite proteolyses host haemoglobin, releasing heme as a by-product. β-Hematin forms as a crystalline cyclic dimer of oxidized heme and is complexed with protein and lipid products as malarial pigment or hemozoin. The function of monocytes and macrophages is severely inhibited after ingestion of hemozoin. Here, the biologically active moieties may be lipoperoxides such as 4-hydroxynonenal (4-HNE) and 15(R, S) hydroxyeicosatetraenoic acid (HETE) produced by oxidation of membrane lipids (Adam, 1990). Their effect on other cellular functions, such as erythropoiesis, has not been established. Anemia in *falciparum* malaria is clearly multifactorial

and there is a strong argument that erythrocyte destruction and ineffective erythropoiesis play equal parts in the etiology of malarial anemia (Weatherall *et al.,* 2002; Gazzinelli *et al.,* 2014).

(e) Respiratory Distress and Metabolic Acidosis: Respiratory distress is common in severe disease and has been shown to be an independent predictor of poor outcome (Kappe, 1999). It is defined by tachypnea, by deep, gasping breathing, and by use of the secondary muscles of respiration and usually represents metabolic acidosis, although acute respiratory infection must be carefully excluded (Chotivanick, 2000). Acidosis is largely due to excessive lactic acid although other anions may contribute to the anion gap Salicylate toxicity can also cause a metabolic acidosis when inappropriate home treatment with aspirin occurs (Zimmerman, 1999).

Some children presenting with respiratory distress are dehydrated and may be resuscitated with saline. However, the majority of children presenting with respiratory distress are severely anemic, have a metabolic acidosis secondary to reduced oxygen-carrying capacity, and respond to rapid transfusion of fresh blood (Zimmerman, 1999). A minority of children with respiratory distress does not respond to appropriate resuscitation. They probably represent a heterogeneous clinical group and may have renal failure, systemic bacterial infection, or a more profound syndrome of systemic disturbance due to malaria parasites (Weatherall *et al.,* 2002; Gazzinelli *et al.,* 2014).

(f) Hypoglycemia: Hypoglycemia is most common in children and in pregnant women with severe disease (Galinski *et al.,* 1992). Hypoglycemia may be evident at presentation or may occur during treatment and sometimes in the face of glucose infusions. In children, insulin levels are appropriate and hypoglycemia appears more likely to follow impaired hepatic gluconeogenesis and increased consumption of glucose in peripheral tissues and by parasites (Rayner *et al.,* 2001). In adults, hypoglycemia has been associated with hyperinsulinemia, and it

has been postulated that malaria-derived products and/or quinine directly or indirectly stimulate the beta cells of the pancreatic islets Galinski *et al*., 1992). Whatever the pathogenesis of the condition, it is clear that all severely ill patients with malaria should be carefully monitored and aggressively treated for hypoglycemia (Weatherall *et al.,* 2002; World Malaria Report, 2013).

2.1.4. Outcome of Malaria Infection

The prognosis of severe falciparum malaria is poor, with a case fatality rate of 15-20% in African children (Keen *et al.,* 1994). Mortality is higher when multiple syndromes of severe disease are present (Keen *et al.,* 1994). A number of clinical and laboratory parameters have been associated with poor outcomes, including deep coma, respiratory distress, hypoglycemia (blood or plasma glucose < 2.2 mM), metabolic acidosis (lactate > 5 mM), raised CSF lactate, renal failure (creatinine > 265 μmol), hyperparasitemia, appearance of pigmented parasitized erythrocytes, and leucocytosis (Keen *et al.,* 1994). Severe malaria in nonimmune adults also has a high case-specific mortality, and here the rate is probably dependent on the availability of critical care facilities to support multiorgan failurure (Preiser *et al.,* 1999).

One unexplained but consistent feature of the epidemiology of clinical malaria is the age distribution of syndromes of severe disease. Children born in endemic areas are protected from severe malaria in the first 6 months of life by the passive transfer of maternal immunoglobulins and by expression of fetal hemoglobin (Preiser *et al.,* 1999). Beyond infancy, the presentation of disease changes from predominantly severe anemia in children aged between 1 and 3 in areas of high transmission to predominantly cerebral malaria in older children in areas of lower transmission (Preiser *et al.,* 1999). In this light, the picture of cerebral malaria with multisystem disease in nonimmune adults represents an extreme end of the spectrum in the relationship between age, transmission, and syndrome of severe disease. The reasons for this pattern are

unknown but probably include age-specific responses to malaria and also cross-reactive responses activated during infection in older children. Many travelers (and returning residents with significant levels of clinical immunity) present with what is effectively mild malaria as seen in African children. However, in nonimmunes, severe disease can progress very rapidly and cause life-threatening illness. In this group, multiorgan failure is more prominent than in children with malaria, and some features of the syndromes of malaria differ from those described above for African children (Okoyeh *et al.*, 1999; WHO, 2015).

(a) Cerebral Malaria: Coma is a very prominent feature of severe illness in nonimmunes, although neurological sequelae are recorded less frequently. However, epilepsy and psychiatric disturbances have been found at increased frequency in a large series of Vietnam veterans who suffered from cerebral malaria (Dolan *et al.*, 1990; WHO, 2015).

(b) Respiratory Failure: Deteriorating respiratory function with widespread pulmonary edema may develop during the disease and carries a very poor prognosis. Some cases of pulmonary edema may be secondary to fluid overload and/or rapid correction of dehydration (Sim *et al.*, 1994). However, other patients have adult respiratory distress syndrome (ARDS) with a normal pulmonary wedge pressure, suggesting that the primary abnormality is endothelial damage and excessive permeability. The primary causes of endothelial damage are unknown (Sim *et al.*, 1994).

(c) Renal Failure: Renal impairment is common in severe malaria in nonimmune adults, and the microscopic pathology is acute tubular necrosis: glomerulonephritis is rare (Mayer *et al.*, 2001). In some cases acute tubular necrosis may be precipitated by intravascular hemolysis. The pathogenesis in other cases is unclear. Patients may require short-term dialysis for acidosis, fluid overload, hyperkalemia, or rapidly rising creatinine (Weatherall *et al.*, 2002).

(d) Bleeding Disorders: Disordered coagulation and clinical evidence of bleeding are not infrequent in adults, and patients may present with bleeding at injection sites, gums, or epistaxis (Weatherall *et al.,* 2002). Other complications include hemoglobinuria secondary to intravascular hemolysis and jaundice which are more common in adults than in children. As in children, concurrent bacteremia is common (Weatherall *et al.*, 2002).

(e) Malaria in Pregnancy: During pregnancy, women are both more susceptible to malaria infection and also more likely to develop hypoglycemia and pulmonary edema. In pregnant women, malaria infection, often without fever, may nevertheless cause anemia and placental dysfunction. This effect is greatest in primigravidae and has been attributed to the adhesion of parasitized erythrocytes to chrondrotin sulfate A and hyaluronic acid in the placenta. Fetal growth is impaired and babies born to women with placental malaria are on average 100g lighter than controls born to women without malaria. The subsequent contribution of malaria to infant mortality is substantial (Patel, 2001).

2.1.5. Clinical features of Malaria Infection

In *P. vivax* and *P. ovale* malaria high parasitaemia is rare, as invasion of erythrocytes is limited to reticulocytes. The parasites do not appear to sequester in the peripheral circulation or cause organ-specific syndromes (Patino *et al.,* 1997). Mortality is limited to occasional deaths from splenic rupture or from intercurrent illnesses in already weakened individuals. Nevertheless, *P. vivax* malaria has been clearly associated with anemia during pregnancy and with low birth weight in children of affected mothers. In these cases, cytokines or other inflammatory mediators appear to cause placental dysfunction (Patino *et al.,* 1997). *P. malariae* infection is also rarely fatal but is distinguished by the persistence of blood-stage parasites for up to 40 years. It can,

however, cause a progressive and fatal nephrotic syndrome (Weatherall *et al.,* 2002; World Malaria Report, 2013).

(a)Changes in White cells due to acute malaria infection: Changes in leucocyte and platelet counts are present in malaria (Weatherall *et al.,* 2002). Malaria is accompanied by a modest leucocytosis, although leukopenia may also occur. Occasionally, leukemoid reactions have been observed. Leukocytosis has been associated with severe disease (English, 1996). A high neutrophil count may also suggest intercurrent bacterial infection. Monocytosis and increased numbers of circulating lymphocytes are also seen in acute infection, although the significance of these changes is not established (Hodder, 1996). However, malarial pigment is often seen in neutrophils and in monocytes and has been associated with severe disease and unfavorable outcome (Triglia, 2000).

There is a significant dysfunction of myeloid cells in malaria. The adhesive phenotypes expressed by falciparum-infected erythrocytes were previously thought simply to permit sequestration of parasites in the peripheral circulation (Adams, 1990). Recent work has illuminated how malaria-infected erythrocytes may modulate the function of macrophages and myeloid dendritic cells through the adhesion of malaria-infected cells to CD36 and/or CD51 on host cells (Adams, 1990). Furthermore, the function of monocytes and macrophages may be inhibited by the action of hemozoin from digested hemoglobin (Braun-Breton, 1992).These observations suggest that the inhibition of phagocytosis and of other inflammatory responses, mediated by adhesion of infected erythrocytes to myeloid cells and by ingestion of hemozoin, may influence the outcome of infection and facilitate survival of both parasite and host (Weatherall *et al.,* 2002).

(b) Changes in Platelets due to acute malaria infection: Thrombocytopenia is almost invariable in malaria and so may be helpful as a sensitive but nonspecific marker of active infection. However, severe thrombocytopenia (< 50×10^9 l) is rare (Weatherall *et al.,* 2002). Increased removal of platelets may follow absorption of immune complexes, but there is no evidence for platelet-specific alloantibodies. By analogy with erythropoiesis, there may be a defect in thrombopoiesis but this has not been established (Weatherall *et al.,* 2002). Thrombocytopenia is not associated with disease severity, although, somewhat paradoxically, platelets have been shown to contribute to disease pathology in animal and in human malaria (Baruch, 1999). Moreover, in human infections, platelets may form 'clumps' with infected erythrocytes (Chen *et al.,* 2000).

(c) Coagulation changes due to acute malaria infection: Abnormalities of laboratory tests of hemostasis, suggesting activation of the coagulation cascades, occur in acute infection, particularly in adults (Marsh, 1995). Patients may present with bleeding at injection sites or mucous membranes. However, histological evidence of intravascular fibrin deposition is notably absent in those dying from severe malaria (Marsh, 1995). However, Factor XIII, normally responsible for cross-linking fibrin, is inactivated during malaria infection, and these data may explain low levels of fibrin deposition in the face of increased procoagulant activity (Newbold, 1999).

2.1.6. Chronic Malaria Infection and the Transmission of Malaria by Transfusion

One of the outstanding problems for those managing the blood supply in non-malaria endemic countries is the prevention of the transmission of malaria by blood transfusion (Weatherall *et al.,* 2002). In nonimmunes, *falciparum* malaria would almost invariably develop within 3 months of return from a malaria-endemic area. Furthermore, the infection is symptomatic at very low levels

of parasitemia. Nonimmune potential blood donors could be safely excluded from transmitting *P. falciparum* if they remain asymptomatic 6 months after return from the tropics, although the exact time limits for exclusion of donors who have returned from the tropics are widely debated (Weatherall *et al.,* 2002). However, malaria is readily transmissible by blood transfusion from semi-immune donors harboring an asymptomatic infection several years after return from a malaria-endemic area (Luse and Miller, 1971). These donors can in principle be identified by the presence of high titer antibodies against blood-stage antigens (Luse and Miller, 1971; World Malaria Report, 2013).

2.1.7. Diagnosis of Malaria Parasites

In spite of the distinct clinical syndromes of severe disease, malaria is often misdiagnosed outside endemic areas as the initial signs of disease are nonspecific (Cooke, 1994). Examination of traditional Giemsa-stained thick or thin films will in most cases confirm the diagnosis (Cooke, 1994). Examination of thick films for malaria parasites is a skill honed by regular practice and probably requires specific training for those working in nonendemic areas (Cooke, 1994). Occasionally, severe disease from malaria can be present without detectable parasites in the peripheral circulation, and empirical therapy should be commenced if the patient is seriously ill and there is clinical suspicion of malaria infection (Weatherall *et al.,* 2002).Early diagnosis and treatment of malaria reduces disease and prevents deaths. It also contributes to reducing malaria transmission (WHO, 2015).

The traditional methods of diagnosis have been challenged but not superseded by the application of modern methods (Ho and White, 1999). Microscopy with fluorescent stains (QBC), polymerase chain reaction assays, and some automated blood cell analyzers offer new approaches, but these are not in wide use (Ho and White, 1999). More recently, dipstick tests for

the detection of parasite antigens HRP2 and pLDH have been developed. These methods may be a useful adjunct in a busy lab, but the tests lose sensitivity at low parasitaemias (< 10,000/μL) and have not been licensed for use by the Food and Drug Administration (Rogerson *et al.,* 1997).

2.1.8. Treatment and Chemotherapy

Malaria requires urgent effective chemotherapy to prevent progression of disease; such chemotherapy may be the most crucial public health intervention to reduce global mortality from malaria (Yipp, 2000). In severely ill patients, good nursing care is vital. Monitoring and treatment of fits and hypoglycemia are essential and antipyretics should be given (Yipp, 2000).

The drug treatment of malaria must take account of the expected pattern of drug resistance in the area where the infection was contracted, the severity of clinical disease, and the species of parasite. The spread of drug-resistant parasites and the optimal use of affordable, effective drugs are of continual concern, and these topics have been reviewed recently (Beeson, 1999; Newbold, 1997). The best available treatment, particularly for *P. falciparum* malaria, is artemisinin-based combination therapy (ACT) (WHO, 2015).

(a) Blood Transfusion: Blood transfusion is in principle a straightforward solution to the treatment of severe malarial anemia, although controversy exists over the trigger for transfusion and the rate of administration of blood (Weatherall *et al.,* 2002). The standard regimes of cautious and slow delivery of blood have been challenged by the demonstration that rapid initial flow rates may correct lactic acidosis. However, in nonimmunes and pregnant women, blood transfusion must be accompanied by careful hemodynamic monitoring to avoid precipitating or exacerbating pulmonary edema (Weatherall *et al.*, 2002).

No formal controlled trials for the transfusion of patients with malaria have been performed. Whatever clinical guidelines emerge, blood transfusion in the heartland of malaria endemic areas

is beset by many practical and theoretical problems, including the absence of well-characterized donor panels and the residual risk of HIV transmission in the serological window of infectivity without detectable antibodies (estimated at 1 in 2000) (Weatherall *et al.,* 2002). At a practical level, positive indirect antiglobulin tests in acute infection may make the exclusion of alloantibodies difficult. Depending on the clinical urgency and transfusion history, the least serologically incompatible blood may have to be given (Weatherall, *et al*., 2002).

(b) Exchange Transfusion: One therapeutic option available in North America and in Europe for the urgent treatment of nonimmune patients with severe disease would be exchange blood transfusion. This procedure removes nonsequestered, infected erythrocytes and possibly circulating 'toxins.' In the absence of evidence from trials for the use of exchange transfusion in malaria, some have suggested that this treatment could be given for hyperparasitemia (> 20%) in severely ill nonimmune patients (Baruch, 1995).

2.1.9. Malaria Prevention

Vector control is the main way to prevent and reduce malaria transmission. If coverage of vector control interventions within a specific area is high enough, then a measure of protection will be conferred across the community. WHO recommends protection for all people at risk of malaria with effective malaria vector control. Two forms of vector control – insecticide-treated mosquito nets and indoor residual spraying – are effective in a wide range of circumstances. Long-lasting insecticidal nets (LLINs) are the preferred form of insecticide-treated mosquito nets (ITNs) for public health programmes. In most settings, WHO recommends LLIN coverage for all people at risk of malaria. The most cost-effective way to achieve this is by providing LLINs free of charge, to ensure equal access for all. In parallel, effective behaviour change communication strategies

are required to ensure that all people at risk of malaria sleep under a LLIN every night, and that the net is properly maintained (WHO, 2015).

Indoor residual spraying (IRS) with insecticides is a powerful way to rapidly reduce malaria transmission. Its full potential is realized when at least 80% of houses in targeted areas are sprayed. Indoor spraying is effective for 3–6 months, depending on the insecticide formulation used and the type of surface on which it is sprayed. In some settings, multiple spray rounds are needed to protect the population for the entire malaria season (WHO, 2015).

Antimalarial medicines can also be used to prevent malaria. For travellers, malaria can be prevented through chemoprophylaxis, which suppresses the blood stage of malaria infections, thereby preventing malaria disease. For pregnant women living in moderate-to-high transmission areas, WHO recommends intermittent preventive treatment with sulfadoxine-pyrimethamine, at each scheduled antenatal visit after the first trimester. Similarly, for infants living in high-transmission areas of Africa, 3 doses of intermittent preventive treatment with sulfadoxine-pyrimethamine are recommended, delivered alongside routine vaccinations (WHO, 2015).

In 2012, WHO recommended Seasonal Malaria Chemoprevention as an additional malaria prevention strategy for areas of the Sahel sub-Region of Africa. The strategy involves the administration of monthly courses of amodiaquine plus sulfadoxine-pyrimethamine to all children under 5 years of age during the high transmission season. Much of the success in controlling malaria is due to vector control. Vector control is highly dependent on the use of pyrethroids, which are the only class of insecticides currently recommended for ITNs or LLINs. In recent years, mosquito resistance to pyrethroids has emerged in many countries. In some areas, resistance to all 4 classes of insecticides used for public health has been detected.

Fortunately, this resistance has only rarely been associated with decreased efficacy of LLINs, which continue to provide a substantial level of protection in most settings. Rotational use of different classes of insecticides for IRS is recommended as one approach to manage insecticide resistance (WHO, 2015).

However, malaria-endemic areas of sub-Saharan Africa and India are causing significant concern due to high levels of malaria transmission and widespread reports of insecticide resistance. The use of 2 different insecticides in a mosquito net offers an opportunity to mitigate the risk of the development and spread of insecticide resistance; developing these new nets is a priority. Several promising products for both IRS and nets are in the pipeline (WHO, 2015).

2.2.1. History of Epstein - Barr virus

The Epstein–Barr virus (EBV) was named after Michael Anthony Epstein (born 18 May, 1921), a Professor Emeritus at the University of Bristol, and Yvonne Barr (born 1932), a 1966 PhD graduate from the University of London, who together both discovered EBV (McGrath, 2014) and, in 1964, published on the existence of the virus (Epstein *et al.*, 1964). In 1961, Epstein, a pathologist and expert electron microscopist, attended a lecture on "The commonest children's cancer in tropical Africa: a hitherto unrecognised syndrome." This lecture, by Denis Parsons Burkitt, a surgeon practicing in Uganda, was the description of the "endemic variant" (pediatric form) of the disease that bears his name. In 1963, a specimen was sent from Uganda to Middlesex Hospital to be cultured. Virus particles were identified in the cultured cells, and the results were published in *The Lancet* in 1964 by Epstein, Bert Achong, and Barr. Cell lines were sent to Werner and Gertrude Henle at the Children's Hospital of Philadelphia who developed serological markers. In 1967, a technician in their laboratory developed mononucleosis and they were able to compare a stored serum sample, showing that antibodies to the virus developed.

(Epstein, 2005; Erle, 2005; Miller, 2006). In 1968, they discovered that EBV can directly immortalize B cells after infection, mimicking some forms of EBV-related infections (Henle and Henle, 1980) and confirmed the link between the virus and infectious mononucleosis (Young, 2009).

Epstein-Barr virusis a member of the herpes virus 4 (HHV-4) family and one of the eight viruses in the herpesviridae family and one of the most common viruses in humans (Maeda *et al.,* 2009). It is a double stranded DNA enveloped virus whose nucleocapsid is 100 nm in diameter with 162 capsomers (Maeda *et al.,* 2009). Its membrane is derived by budding of immature particles through cell membrane and it is required for infectivity. The genome is a linear double stranded DNA molecule with 172 kbp (Maeda *et al.,* 2009). The viral genome does not normally integrate into the cellular DNA but forms circular episomes which reside in the nucleus. The genome is large enough to code for 100-200 proteins but only a few have been identified (Ebell, 2004; Hammerschmidt and Sugden, 2013).

Epstein-Barr virusis the most common and most successful human virus (Ebell, 2004). EBV occurs worldwide, affecting anyone at any point in their lifetime (Ebell, 2004). Once infected the virus stays with the person for the rest of their life (Ebell, 2004). The significance of this virus is that apart from its most common infection which is infectious mononucleosis, EBV has also been associated with many other diseases (Ebell, 2004). Burkitt lymphoma is a common tumor among children in parts of sub-Saharan Africa, including Uganda (Wabinga, *et al.,*2000). Marked seasonal, temporal and geographical variations in incidence, with evidence of space-time clustering, have suggested an infectious etiology (Morrow*et al.,* 1982; Hammerschmidt and Sugden, 2013).

Particular interest has focused on the role played by two infectious agents—the Epstein-Barr virus and malaria (Morrow*et al.,* 1976;De-Thé,1977). On the basis of the geographical distributions, holoendemic malaria has been suggested as a cofactor for Burkitt lymphoma (Carpenter *et al.,* 2008). Associations with socio-economic and other environmental factors have also been suggested but comparatively little studied (Morrow*et al.,* 1982) and the potential role played by maternal characteristics, such as a mother's exposure to infectious diseases, have yet to be addressed (Carpenter *et al.,* 2008).

Most reviews state that >90% of adults are antibody positive by the age of 35 years (Levin *et al.,* 2005). Seroepidemiological studies have shown that about 91% of the adults worldwide have had first time infection by EBV (Serraino*et al.,* 2005). EBV infects >95% of the global population with prevalence varying by sociodemographics and region (Svahn*et al.,* 2016). In contrast, the prevalence of EBV antibodies among children is lower, varying from 20% to 80% depending on age and geographic location (Hjalgrim *et al.,* 2007). Factors implicated in early acquisition of primary EBV infection include geographic region (Hjalgrim *et al.,* 2007), socioeconomic status, crowding or sharing a bedroom (Crowcroft *et al.,* 1998) maternal education level (Figueira-Silva *et al.,* 2004), day care attendance (Hesse, 1983), and school catchment area (Crowcroft *et al.,* 1998). High levels of antibodies to EBV are found in all African children with BL, but in only half of normal African children. The reason for this association remains largely unknown (Jain*et al.,* 2011). The virus may be a prime etiologic agent, a co-carcinogen, or just an innocent passenger. However, it has been suggested that malaria infection in Africa may impair cellular immunity to EBV and induce polyclonal B-cell activation with an expansion of EBV-infected B cells. These changes may enhance the proliferation of B cells, increasing the likelihood of a c-myc translocation-the hallmark of BL (Jain *et al.,* 2011).

Most people become infected with EBV and gain adaptive immunity. Approximately 95% of the world's population sustains an asymptomatic life-long EBV infection. EBV persists in the memory B-cell pool of normal healthy individuals and any disruption of this interaction results in virus-associated B-cell tumors.Infants become susceptible to EBV as soon as maternal antibody protection disappears. Many children become infected with EBV, and these infections usually cause no symptoms or are indistinguishable from the other mild, brief illnesses of childhood. When infection with EBV occurs during adolescence or teenage years, it causes infectious mononucleosis 35-69% of the time (Jain *et al.*, 2011).

There appears to be a complex interplay between age of acquisition of primary EBV infection, symptomatic versus asymptomatic infection, and the risk of EBV-associated cancers or autoimmune diseases (Thacker *et al.,* 2006). Identifying demographic risk factors for age-specific acquisition of the virus and describing EBV infection disparities on the basis of race/ethnicity and/or country of birth will improve our current understanding of EBV infection in children and could inform appropriate prevention strategies (Balfour *et al.,* 2012).

Co-infection with *Plasmodiumfalciparum* malaria and Epstein-Barr virus (EBV) is a major risk factor for endemic Burkitt lymphoma (eBL), still one of the most prevalent pediatric cancers in equatorial Africa (Chattopadhyay*et al.,* 2012). First described in 1958 (Burkitt, 1958), endemic Burkitt lymphoma (eBL) remains one of the most prevalent childhood cancers in equatorial Africa. The average annual incidence is 2 per 100,000 children, with a peak age range of 5 to 9 years (Parkin *et al.,* 2008). Epstein-Barr virus was discovered in a tumor sample obtained from a patient with eBL (Epstein *et al.,* 1964), and EBV DNA has subsequently been detected in tumor cells from 95% of eBL cases (Pagano, 1999). Thus, EBV was identified as the first human tumor virus, with ensuing studies revealing the virus-mediated oncogenic processes (Thorley-Lawson

and Allday, 2008). However, eBL is most common in children residing in areas with the highest malaria transmission intensities; an enigmatic observation that leaves the malaria-associated mechanisms involved in the etiology of eBL insufficiently established by comparison (Rainey *et al.,* 2007).

Infection with EBV occurs early in most African populations, and almost 100% of children are EBV-seropositive by 3 years of age (De The,1975). Primary infection during childhood is typically asymptomatic, whereas infection in young adults can result in acute infectious mononucleosis (AIM), a self-limited lymphoproliferative disorder. To date, most immunologic studies of EBV infection are based on healthy seropositive adults or cases of AIM among adolescents in Europe or the United States (Hislop *et al.,* 2007). Collectively, these studies show that $CD8^+$ cytotoxic T lymphocytes (CTL) are necessary for immune surveillance and control of persistent EBV infection (Khanna and Burrows, 2000). The CTL response to EBV is directed against an array of antigens expressed during the lytic and latent phases of the viral life cycle (Khanna and Burrows, 2000), and control is associated with HLA class I-restricted gamma interferon (IFN-γ) responses (Yang *et al.,* 2000). Previous studies have also demonstrated phenotypic and functional heterogeneity among EBV-specific $CD8^+$ T-cell populations (Hislop *et al.,* 2001).

2.2.2. EBV Structure, Genome and Life Cycle

Epstein-Barr virus has a double stranded DNA with no RNA stage (Cohen, 2000). It belongs to the Herpesvirales; Herpesviridae (family); Gammaherpesvirinae (subfamily); Lymphocryptovirus (genus); Human herpesvirus 4 (species) (Cohen, 2000). The virus is approximately 122 nm to 180 nm in diameter and is composed of a double helix of DNA of about 172,000 base pairs and containing about 85 genes (Amon, 2004). The DNA is surrounded

by a protein nucleocapsid. This nucleocapsid is surrounded by a tegument made of protein, which in turn is surrounded by an envelope containing both lipidsand surface projections of glycoproteins which are essential for infection of the host cell(Odumadeand Hogquist, 2011).

The structure of EBV (Plate I) is an icosahedral nucleocapsid (Ebell, 2004). The nucleocapsid is composed of 162 capsomers. The nucleocapsid is then surrounded by a protein outer covering which is then surrounded by a viral envelope that contains numerous glycoproteins (Ebell, 2004).Replication of Epstein-Barr virus can occur in two ways: infection of skin cells that result in lysis of host cells and release of virions and Primary B-cell that results in latency infection (Cohen, 2000; Kieff and Rickinson, 2007). During the replication stage, the virus constantly replicates due to the EBV DNA forming an episome, which becomes circular and joins together, causing continuous replication (Kieff and Rickinson, 2007).

Human herpesviruses comprise three subfamilies: (i) herpesviruses [herpes simplex viruses (HSV) 1 and 2 and varicella zoster virus (VZV)], (ii) herpesviruses [cytomegalovirus (CMV) and human herpesvirus (HHV) 6 and 7 and] (iii) herpesviruses, comprising the Kaposi's sarcoma associated herpesvirus (KSHV or HHV8) and Epstein–Barr virus (EBVor HHV4) (Tarbouriech *et al.*, 2006). The last family infects the vast majority of the world's human population, establishing and maintaining a lifelong persistence in the infected host (Tarbouriech *et al.*, 2006). Primary infection typically occurs in childhood and is frequently asymptomatic. In contrast, a delayed primary infection in adolescents or young adults results in infectious mononucleosis (IM) in approximately half of cases, with symptoms including fever, pharyngitis, lymphadenopathy and splenomegaly (Tarbouriech *et al.*, 2006).

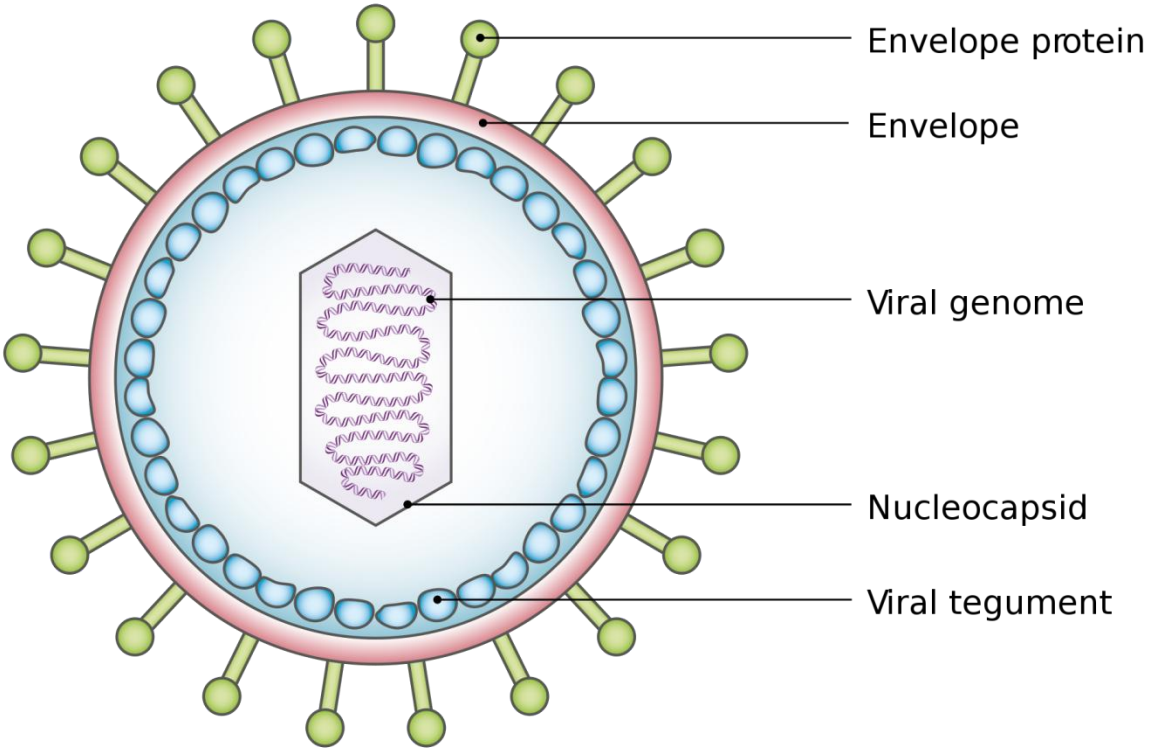

Figure2.1: Structure of the Epstein-Barr virus (Ebell, 2004).

Infectious mononucleosis (IM) is a self-limiting lymphoproliferative disorder characterized by an expansion of EBV-infected B-lymphocytes associated with viral lytic replication in the oropharynx, controlled by a vigorous CD8+ cytotoxic T-cell immune response. The majority of cases of acute IM recover, but serious complications can occasionally lead to death (Tarbouriech *et al.,* 2006). Epstein-Barr virus is associated with a number of cancers in the immunocompetent host, particularly Burkitt's lymphoma and nasopharyngeal carcinoma, which are endemic in African and Asian populations (Raab-Traub, 2005). Furthermore, EBV can lead to immunoproliferative disease in immunosuppressed patients, notably those infected with HIV (Rickinson and Kieff, 1996). Currently licensed antiherpesvirus drugs (acyclovir and related compounds) directed against viral DNA synthesis show little effect against EBV (Coen and Schaffer, 2003).

Epstein-Barr virus is composed of an inner capsid that contains the viral double-stranded DNA genome, surrounded by a membrane carrying various surface glycoproteins (Tarbouriech *et al.,* 2006). Tegument fills the space between the capsid and the membrane. During the latent stage of infection in B-lymphocytes a very limited set of proteins is expressed (Tarbouriech *et al.,* 2006). The viral DNA forms a circular episome which is associated with the cellular chromosomes and is replicated by the cellular machinery during cell division. After activation, the infection can switch to the lytic cycle, leading to the expression of the full set of viral proteins and production of viral particles. This complex lifestyle utilizes about 86 predicted proteins, meaning that EBV has one of the largest genomes of human viruses (Tarbouriech *et al.,* 2006). The principal viral functions are receptor binding and cell entry, maintenance of latency, nucleotide metabolism, DNA replication and packaging and capsid assembly (Tarbouriech *et al.,* 2006). EBV also codes for a number of immune-modulators. Some little-studied proteins shuttle viral particles from the

nucleus, the site of viral replication, to the extracellular space and a number of proteins still have no assigned function (Tarbouriech *et al.,* 2006).

The Epstein-Barr virus is a γ-herpesvirus that infects B cells and epithelial cells and that has been linked to malignancies in both cell types in vivo (Matsuura *et al.,* 2010). EBV, like other herpesviruses, has three glycoproteins, glycoprotein B (gB), gH, and gL, that form the core membrane fusion machinery mediating viral penetration into the cell. The gH and gL proteins associate to form a heterodimeric complex, which is necessary for efficient membrane fusion and also implicated in direct binding to epithelial cell receptors required for viral entry (Matsuura *et al.,* 2010).

The glycoprotein structure is comprised of four domains organized along the longest axis of the molecule. The gL subunit and N-terminal residues of gH form a globular domain at one end of the structure and it is involved in interactions with gB and activation of membrane fusion. The C-terminal domain of gH, proximal to the viral membrane, is also implicated in membrane fusion (Matsuura *et al.,* 2010). The gH/gL structure locates an integral binding motif, implicated in epithelial cell entry, on a prominent loop in the central region of the structure. Multiple regions of gH/gL, including its two extreme ends, are functionally important, consistent with the multiple roles of gH/gL in EBV entry (Matsuura *et al.,* 2010).

The Epstein-Barr virus uses different pathways for the infection of epithelial cells and B lymphocytes. For both cell types, the minimal viral glycoprotein components that mediate membrane fusion have been identified (Hutt-Fletcher, 2007). As with other herpesviruses, EBV uses the core viral entry glycoproteins, glycoprotein B (gB) and the gH/gL complex. For the infection of B lymphocytes, EBV requires an additional protein, gp42, which binds to host human leukocyte antigen (HLA) class II molecules, triggering the membrane fusion step (Hutt-Fletcher, 2007). The gp42 has multiple functional sites for interaction with gH/gL, HLA class II,

and potentially, another unknown binding ligand that could be engaged through a large surface-exposed hydrophobic pocket (Shaw *et al.,* 2010). The gp42 protein binds to the gH/gL complex with nanomolar affinity through its N-terminal region, and this interaction can be recapitulated with a synthetic peptide of ~35 amino acid residues (Kirschner *et al.,* 2009). EBV glycoprotein-mediated membrane fusion with epithelial cells does not require gp42 but only gB and gH/gL, and fusion can be completely blocked by saturating amounts of either gp42 or short gp42-derived peptides (Liu *et al.,* 2010), consistent with the hypothesis that gp42 levels in the virion regulate the cellular tropism of the virus in vivo (Borza and Hutt-Fletcher, 2002). Recent observations indicate that EBV gH/gL engages integrins αvβ6 and/or αvβ8 on epithelial cells to trigger membrane fusion and entry (Chesnokova, 2009).

2.2.3. EBV Replication

EBV is an enormously successful human parasite, having infected more than 6.5 billion people in the world today (Hammerschmidt and Sugden, 2013). Its success is intimately tied to its having evolved as an extrachromosomal replicon, inducing the infected cell to cycle, and successfully usurping the cell's machinery to carry out its licensed DNA synthesis. It has built on a common herpesviral property of amplifying viral DNA during productive infections through dedicated viral origins of DNA synthesis and an array of viral proteins that mediate this unlicensed synthesis. It carries out these different modes of DNA replication in human B cells by affecting the differentiated state of it B-cell host (Hammerschmidt and Sugden, 2013).

2.2.3.1. (a) Attachmentto B cells: Eight virus glycoproteins have been implicated in some way in EBV entry into either a B cell or an epithelial cell. One of the most abundant of these in the virus envelope, gp350/220 (Johannsen *et al.,* 2004), is responsible for attachment of the virus

with high affinity (Moore *et al.,* 1989) to the complement receptor type 2 (CR2) on B cells (Nemerow *et al.,* 1985; Hutt-Fletcher, 2007). An EBV recombinant lacking gp350/220 can transform B cells with much-reduced efficiency (Janz *et al.,* 2000). The CR2 is perhaps not the only portal by which the virus can access a B cell. It is, however, clearly the predominant one (Hutt-Fletcher, 2007). Antibodies to gp350/220 that block virus binding neutralize B-cell infection, and soluble forms of either CR2 or gp350/220 can do the same (Moore *et al.,* 1991).

The extracellular domain of CR2 is composed of tandem repeats of structural modules 60 to 75 amino acids in length known as short consensus repeats (SCR), and the binding site for gp350/220 has been genetically mapped to a region composed of residues from the membrane distal SCR1 and SCR2 (Martin *et al.,* 1991). Attachment initially positions the virus at approximately 50 nm from the cell surface (Nemerow and Cooper, 1984), but the segmental flexibility that the repeated SCRs may give to CR2 and the possibility of an exchange in binding from gp350 to gp220 may contribute to moving the virus closer to the cell membrane (Weisman *et al.,* 1990; Hutt-Fletcher, 2007).

Binding of gp350/220 also triggers capping of CR2 and endocytosis of the virus (Nemerow and Cooper, 1984; Tanner *et al.,* 1987). The CR2 can function as a signal transducer both independently and as part of a signal transduction complex that includes cluster of differentiation 19 (CD19) and CD35 (Fearon and Carter, 1995). Cross-linking by gp350/220 activates necrotic factor, NF-κB (Sinclair and Farrell, 1995; Sugano *et al.,* 1997) and induces interleukin-6 via a protein kinase C pathway (Tanner *et al.,* 1996;D'Addario *et al.,* 2014).

(b) Attachment to Epithelial cells: Attachment of the virus to an epithelial cell is a more-complicated affair. CR2 is expressed at least at low levels on some epithelial cells in culture (Fingeroth *et al.,* 1999) in the absence of CD19 and CD35, and infection of an epithelial cell that

has been engineered to express high levels of CR2 can be very efficient (Borza *et al.*, 2004). Notably, in this regard, EBV-mediated cross-linking of CR2 on epithelial cells, which lack the other components of the CR2 signaling complex found on B cells, stimulates relocalization and clustering of CR2 and the forming homolog over-expressed in spleen (Gill *et al.*, 2004). Formins are molecular scaffolds that nucleate actin, linking signal transduction to actin reorganization and gene transcription (Gill *et al.*, 2004), and this may contribute to successful delivery of the virus to the nucleus of the cell. However, which, or even whether, epithelial cells normally express CR2 in vivo remains uncertain. Studies with monoclonal antibodies to CR2 were compromised by the finding that the antibody most commonly used cross-reacted with an unrelated epithelial cell protein (Young *et al.*, 1989).

At least three other possible attachment mechanisms have been proposed that involve neither gp350/220 nor CR2 (Sixbey and Yao, 1992). The first was a demonstration that virus coated with immunoglobulin A specific to gp350/220 can bind productively to the polymeric immunoglobulin-A receptor (Sixbey and Yao, 1992). This may be particularly relevant to infection via the basolateral surface of an epithelial cell in an immune host, although, since in polarized cells virus was transported intact from the basolateral to the apical surface; it may be more relevant to trans-epithelial transport than direct infection (Gan *et al.*, 1997). The second was a demonstration that in the absence of CR2 a complex of two additional glycoproteins, gH and gL, can serve as epithelial ligands (Miller and Hutt-Fletcher, 1988;Oda *et al.*, 2000).

Epstein-Barr virusgH, a type 1 membrane protein, is, like its homologs in other herpesviruses, misfolded and retained in the endoplasmic reticulum in the absence of the much smaller type 2 protein, gL (Li *et al.*, 1997). No separate function in addition to facilitating folding and transport of gH has been ascribed to gL, and the two proteins, which associate noncovalently, are usually

referred to as a unit, the gHgL complex. EBV derived from a B cell can bind well to a CR2-negative epithelial cell, but recombinant viruses that lack gHgL lose this ability (Molesworth *et al.,* 2000). A soluble form of gHgL made in baculovirus can bind specifically to epithelial cells, but not B cells and its binding can be reduced by a monoclonal antibody specific for the gHgL complex (Borza *et al.,* 2004). The same antibody can also reduce virus binding (Molesworth *et al.,* 2000).

Finally, and most intriguing, on polarized epithelial cells, which presumably resemble more closely the environment that the virus encounters in vivo, an interaction between a multispan virus membrane protein encoded by the open reading frame and integrins has been demonstrated (Tugizov *et al.,* 2003). Antibodies to integrins and to a reading-frame fusion protein only partially block binding to polarized epithelial cells but have a significant impact on infection via the basolateral surface of the polarized monolayer (Xiao *et al.,* 2007). The protein is not required for cell-cell fusion (McShane, and Longnecker, 2004). However, whether the reading-frame fusion protein integrin interaction is primarily responsible for attachment or whether it is most relevant to post attachment events to which signaling may make an important contribution is not yet clear (Johannsen*et al.,* 2004).

2.2.3.2.Fusion and Penetration: Fusion of the EBV envelope with both a B cell and an epithelial cell requires three glycoproteins, gH, gL and gB, which are conserved throughout the herpesvirus family and which have been referred to as the core fusion machinery (Spear and Longnecker, 2003). However, how and where this machinery is activated differs significantly for each cell type (Spear and Longnecker, 2003).

(a) Fusion and Penetration of B cells: Fusion of the virus with a normal B cell requires endocytosis, which is triggered by the interaction between gp350/220 and CR2 and occurs in a low-pH compartment, although a low pH is not required (Miller and Hutt-Fletcher,1992). The virus proteins that are both necessary and sufficient for efficient fusion are gH, gL, gB, and gp42. A role for gH was first demonstrated when it was shown that virosomes made from proteins depleted of those that bound to an antibody to gH could attach to B cells but could not fuse (Haddad and Hutt-Fletcher, 1989). It was later confirmed by analysis of the phenotype of a recombinant gH-null virus (Molesworth *et al.,* 2000). A role for EBV gB was not possible to evaluate genetically, since, unlike its homologs in other herpesviruses, it is essential for virus morphogenesis and egress (Herrold *et al.,* 1995). However, when cell-cell fusion assays were developed as models of virus-cell fusion, it became apparent that its contribution is also very important (Haan *et al.*,2001).

The fourth and final protein involved in fusion of EBV with a B cell, gp42, has homologs only among the lymphocryptoviruses (Rivailler *et al.,* 2002). It is another type 2 membrane protein, and it associates noncovalently with gHgL. The original observation was that gp42 resembles a C-type lectin and that a soluble form made by replacing the signal sequence with one that is efficiently cleaved can bind to HLA class II (Spriggs *et al.,* 1996). Within the three-part complex of gHgLgp42 in the virus, this interaction is now thought to proviDe The trigger for B-cell fusion. A monoclonal antibody that was initially mapped to gH (Strnad*et al.,* 1982) but ultimately proved to react with gp42 (Li *et al.,* 1997) inhibited interaction with HLA class II (Li *et al.,* 1995) and also blocked virus-cell fusion (Miller and Hutt-Fletcher,1988).

Reciprocally, a monoclonal antibody to HLA class II that blocked interaction with gp42 also inhibited infection. The soluble form of gp42 initially used to document HLA class II binding

could inhibit infection by competing with gp42 in the virus, and B cells that lacked HLA class II could be efficiently infected only if HLA class II expression was restored (Li *et al.,* 1997). A recombinant gp42-null virus could infect a B cell only if the cells and the bound virus were treated with an exogenous fusogen such as polyethylene glycol (Wang and Hutt-Fletcher,1998) or if the soluble form of gp42, which retained a gHgL binding domain at its amino terminus, was added in *trans* to reform three-part complexes (Wang*et al.,* 1998; Hammerschmidt and Sugden, 2013).

Binding of gp42 to HLA class II shows some allelic specificity in that nonfunctional HLA-DQ alleles have been identified (Haan *et al.,* 2001), but since all three alleles, HLA-DP, HLA-DQ, and HLA-DR, can be used, it is perhaps unlikely that this has a major impact in an outbred human population. A crystal structure of the ectodomain of gp42 liganded to HLA-DR1 has been solved (Mullen *et al.,* 2002). The protein is most closely related to natural killer cell receptors, such as the murine receptor LY49, which interact with major histocompatibility complex (MHC) class I molecules, but it does not use the canonical surface site which many C-type lectins use to bind to their ligands. Instead, this site forms a hydrophobic pocket that, it is suggested, may be used to accommodate another virus protein or another cell protein important for virus entry perhaps after triggering by HLA class II (Mullen *et al.,* 2002). This is consistent with the observation that mutations in the hydrophobic pocket do not affect HLA class II binding but inhibit fusion (Silva *et al.,* 2004). The contact region on HLA-DR1 is within the β1 domain to one side of the peptide binding groove. It was thought on theoretical grounds to be likely to interfere sterically with the binding of T-cell receptors to the peptide MHC complex and has been shown experimentally to reduce T-helper-cell recognition of B cells (Ressing *et al.,* 2005).

Both peptide-loaded and immature MHC class II proteins that have not yet trafficked to the peptide-loading compartment can interact with gp42 (Ressing *et al.,* 2005), and, as discussed below, this has proven to have an interesting impact on virus tropism. Whether or not additional signaling events may be initiated as a result of a gp42-HLA class II interaction is not yet known, but since signaling via HLA class II molecules is well documented under other circumstances, this remains a reasonable possibility (Watt, 1997).

(b) Fusion and Penetration of Epithelial cells: Fusion with a normal epithelial cell, at least one that is nonpolarized, takes place at neutral pH and does not appear to require endocytosis (Borza *et al.*,2004). Epithelial cells do not constitutively express HLA class II, and thus, not surprisingly, infection is not blocked by the monoclonal antibody that blocks gp42 and HLA class II interactions. A gp42-null virus can infect an epithelial cell as well as, or even better than, wild-type virus. However, not only is gp42 dispensable for epithelial cell infection, but its presence is actually inhibitory (Wang *et al.,* 1998).

Stoichiometric analysis of gHgL and gp42 in the virion indicated that wild-type EBV carries far more gHgL than gp42, implying that there are two kinds of gH complexes, first, the three-part complex composed of gHgLgp42 and the second, a two-part complex composed of only gHgL (Borza *et al.,* 2004). Only three-part complexes can mediate B-cell infection, but conversion of all two-part complexes to three-part complexes by the addition of soluble gp42 in trans blocks epithelial cell infection. This was interpreted to mean that fusion with an epithelial cell is triggered by a direct interaction between gHgL and a novel epithelial cell surface molecule (Borza *et al.,* 2004).

Three monoclonal antibodies that interact with gH or gHgL and have no effect on B-cell infection can also inhibit infection of epithelial cells, whether virus is bound via gp350/220 to

CR2 or via gHgL to gHgLR (Borza *et al.,* 2004). One of them blocks gHgL binding to gHgLR, suggesting that the cell molecule that triggers fusion might be the same protein, gHgLR, which can serve as an attachment receptor in the absence of CR2. Consistent with this is the observation that soluble gp42 also blocks binding of the virus to gHgLR. Use of gHgL for attachment as well as fusion does, however, seem to compromise infection (Borza *et al.,* 2004). The virus can bind efficiently to gHgLR on a CR2-negative epithelial cell, but infection levels are low, either because fusion becomes less efficient or perhaps because engagement of gHgLR does not induce a downstream event required for completion of transport of the virus genome to the nucleus (Borza *et al.,* 2004).

Beyond this, epithelial cell penetration also requires higher levels of gB than does B-cell penetration (Neuhierl,*et al.,* 2002), presumably reflecting a need for more gB in fusion (McShane and Longnecker, 2004). However, the mechanics of fusion itself remain obscure for all herpesviruses (Omerovic *et al.,* 2005). Mutations in an amino-terminal region of gH that is predicted to form a coiled coil significantly reduced fusion in a cell-cell fusion assay (Omerovic *et al.,* 2005), and insertion or point mutations in a region close to the transmembrane domain can either differentially affect B-cell and epithelial cell fusion or abrogate fusion with either cell type (Wu *et al.,* 2005;Wu and Hutt-Fletcher, 2007).

However, some epithelial cell fusion can be mediated in the absence of gHgL by a gB construct with a mutation in the cytoplasmic tail. This has been interpreted to mean that gB may be the critical fusion protein of EBV (Heldwein*et al.,* 2006). Certainly the crystal structure of the homolog of gB in herpes simplex virus shows structural homology with the vesicular stomatitis virus G protein, which is the virus fusogen (Heldwein*et al.,* 2006). Both form trimers characterized by an alpha-helical coiled-coil core and extended β-hairpins that are characteristic of class I and class II fusion proteins, respectively (Hutt-Fletcher, 2007). At this point, however,

perhaps the most that can be definitively said is that virus fusion with a B cell requires a trigger that is transmitted via gp42 to gHgL and gB, whereas fusion with an epithelial cell requires a trigger that is transmitted directly to gHgL. Whether there is a temporal cascade of events culminating in fusion mediated by gB or whether gHgL and gB coordinately contribute to the process remains to be determined (Hutt-Fletcher, 2007).

(c) Transfer of Virus between B Cells and Epithelial Cells: The observation that two-part gHgL and three-part gHgLgp42 complexes are mutually exclusive for epithelial and B-cell entry suggested that the relative amounts of each complex in the virion might impact virus tropism (Hutt-Fletcher, 2007). Analyses of viruses made in B cells and epithelial cells confirmed this. In a B cell, some three-part gHgLgp42 complexes prove to interact with immature MHC class II in the endoplasmic reticulum and are targeted to the HLA class II trafficking pathway, where they are vulnerable to degradation. This does not happen in an HLA class II-negative epithelial cell (Hutt-Fletcher, 2007).

Epithelial cell viruses are, as a result, rich in complexes containing gp42 and are as much as two orders of magnitude more infectious for a B cell than viruses produced by a B cell. B-cell viruses, lower in three-part complexes but rich in two-part complexes, are better able to infect an epithelial cell, although the phenotype is not nearly as striking (Hutt-Fletcher, 2007). This suggested a model of persistent infection in which virus reactivating from a latently infected memory B cell as it terminally differentiates into a plasma blast is equipped to infect an epithelial cell, whereas virus amplified in an epithelial cell is strongly B cell tropic (Hutt-Fletcher, 2007).

Epstein-Barr virus is an orally transmitted virus, and the switch in tropism is effected by levels of gp42, which could impact transmission (Borza *et al.*, 2004). This raised the question of the cellular origins of viruses that are shed in saliva. Viruses made in a B cell, low in gp42, have

been shown to be able to bind to gHgLR on an epithelial cell almost as well as they can bind CR2 on a B cell (Borza *et al.,* 2004). In contrast, virus made in an epithelial cell, high in gp42, binds gHgLR very poorly (Borza *et al.,* 2004). A comparison of the differential abilities of viruses from saliva and viruses from transformed B cells obtained from a series of EBV-positive donors facilitated the conclusion that the majority of viruses shed in saliva are derived from HLA class II-negative cells (Jiang *et al.,* 2006).

For the most-efficient transmission, this either means that the virus directly accesses a B cell in the oral mucosa or that transmission to an epithelial cell for amplification before accessing a B cell is influenced by other events (Turk *et al.,* 2006). Two possibilities have been proposed, and both implicate the differential use of gp350/220; first, antibodies to gp350/220, which inhibit infection of a B cell dependent on CR2 for entry, can enhance infection of an epithelial cell (Turk *et al.,* 2006). The effect is not mediated by Fc receptor binding but is further increased by antibody cross-linking, which may patch gp350/220 in the virus envelope. Saliva from EBV-seropositive individuals has similar effects that can be reversed by depletion of antibodies. The interpretation of these results was that, if gp350/220 is not absolutely required, then its presence may impede access of other essential proteins to the cell surface (Hutt-Fletcher, 2007). Antibodies to gp350/220, an abundant and immunogenic protein, can overcome this in the saliva of an immune host to facilitate re-infection of an epithelial cell or to facilitate transmission through the epithelium in a new host (Hutt-Fletcher, 2007).

Also, there are long-standing observations that epithelial infection is more efficient if epithelial cells are co-cultivated with B cells that are making the virus (Imai *et al.,* 1998). A novel approach to investigation of the phenomenon demonstrated that binding EBV to a B cell before adding both to epithelial cells significantly enhanced infection of the epithelial cells, even in the absence of internalization and infection of the B cell (Shannon-Lowe*et al.,* 2006). The

mechanism of this B-cell transfer is uncertain but intriguing. The gp350/220 is inhibitory to direct epithelial cell infection and the gp350/220 interaction with CR2 on the B cells unmasks envelope components necessary for epithelial cell infection. This parallels the hypothesis for enhanced infection in the presence of antibodies to gp350/220 in saliva (Hutt-Fletcher, 2007).

(d) Post-fusion Events: There is very little concrete information about what happens between fusion and the arrival of the virus genome in the nucleus, only speculation based on what is known for other herpesviruses (Hutt-Fletcher, 2007). Signaling events triggered by interactions between the virus envelope and cell proteins have important consequences for post-fusion events, but how they may impact intracellular transport and uncoating of the virion is a subject that has not yet been substantively addressed (Hutt-Fletcher, 2007).

Two glycoproteins in the virus envelope have, however, also been tentatively implicated in what are presumably immediate post-fusion events that may perhaps have no connection to cell signaling. These are glycoprotein gN, a small type I membrane protein, and glycoprotein gM. Glycoprotein gM is a multispan membrane protein with a long, highly charged cytoplasmic tail rich in prolines and replete with potential phosphorylation sites that might provide a mechanism for the regulation of protein-protein interactions (Lake and Hutt-Fletcher, 2000). Glycoproteins gN and gM are codependent for expression and play important roles in virus assembly and acquisition of the final virus envelope (Lake and Hutt-Fletcher, 2000; Hammerschmidt and Sugden, 2013).

Only very little enveloped virus is made by a recombinant EBV that lacks gN and gM. However, that enveloped virus which is made and is able to bind specifically to CR2 on a B cell is impaired in infection in a way that cannot be rescued by addition of exogenous fusogens such as polyethylene glycol (Hutt-Fletcher, 2000). One hypothesis is that a complex that is necessary for assembly of an enveloped particle when a tegumented capsid associates with a membrane is also

essential to its disassembly when a tegumented capsid must disassociate from a membrane with which its envelope has fused (Hutt-Fletcher, 2000).

2.2.3.3. Self-Assembly of Epstein-Barr virus Capsids: Herpesviruses assemble icosahedral capsid structures in the nuclei of infected cells and six proteins are required for assembly of these structures (Steven and Spear, 1996). The capsid shell is largely composed of multimers of the major capsid protein, which are linked by the triplex structure (trimer of the two triplex proteins) and decorated by the small capsid protein. The internal scaffold protein directs the closure of the shell into an icosahedral structure (Steven and Spear, 1996). The EBV serine protease is synthesized as a 605-amino-acid polyprotein, which cleaves itself between amino acids Ala235 and Ser236 (release [R] site) and also between amino acids Ala568 and Ser569 (maturation [M] site). The catalytic domain resides in the N-terminal 235 amino acids (Donaghy and Jupp, 1995). The six EBV capsid proteins are BcLF1 (major capsid protein), BORF1 (triplex 1), BDLF1 (triplex 2), BdRF1 (scaffold protein), BVRF2 (protease), and BFRF3 (small capsid protein) (Brandon *et al.,* 2009). BVRF2 and BdRF1 are synthesized from the same open reading frame (ORF), and consequently BdRF1 overlaps the C-terminal 261 to 605 amino acids of BVRF2 (Brandon *et al.,* 2009).

Protein-protein interactions drive the self-assembly of herpesvirus capsid structures (Adamson *et al.,* 2006). Capsid assembly for herpesviruses begins in the cytoplasm. Cellular localization studies show that the major capsid protein is transported into the nucleus by the scaffold protein (not cleaved at the maturation site), and triplex protein 2 is transported efficiently in the presence of triplex protein 1 (Adamson *et al.,* 2006). The full-length protease can also interact with the major capsid protein, and this may be one mechanism it uses to localize to the preassembly complex (Sheaffer *et al.,* 2000). The small capsid protein requires both the major capsid and the

scaffold protein for its concentration in the nuclear assembly sites (Rixon *et al.,* 1996). Complexes formed between the two triplex proteins-between the major capsid protein, scaffold protein, and probably the small capsid protein-come together in the nucleus to form the capsid shell (Spencer *et al.,* 1998; Hammerschmidt and Sugden, 2013).

The first closed structure formed in the nucleus is the spherical procapsid, which serves as the substrate for the association with the DNA packaging complex and is filled with a genome complement of DNA (Rixon and McNab, 1999). Within this procapsid, the protease cleaves the scaffold protein at the C terminus, which allows the scaffold protein to be released from the shell during DNA packaging (Gao *et al.,* 1994). Thus, the protease is essential for the maturation of this structure from that containing a scaffold to one containing DNA (Gao *et al.,* 1994). The assembly of herpesvirus capsids and determination of their structures are biased toward the studies of herpes simplex virus (HSV) capsids. Very little information on EBV capsid structure or the molecular pathways of the assembly process is available because of a lack of a tractable system to isolate EBV capsids in quantities sufficient for biochemical and structural analyses (Brandon *et al.,* 2009).

2.2.4. Epidemiology of EBV

Epstein-Barr virus has been detected in all populations and all areas of the world (IARC, 1997), but with noticeable geographical variation in the distribution of EBV genotypes. Two major types of EBV, type 1 and 2, have been described in humans, varying in the genes that encode some of the nuclear proteins in latently infected cells (Sample *et al.,* 1990). Both types are detected all over the world, with type 1 being the most prevalent (Gratama and Ernberg, 1995).Epstein-Barr virus is an ancient virus, and has probably coevolved with its different hosts over the last 90-100 million years (McGeoch *et al.,* 1995). With the ability to establish lifelong

latency and intermittent reactivation after primary infection and with limited clinical symptoms in the majority of infected individuals, EBV has become ubiquitous in all human populations (McGeoch *et al.*, 1995; Hammerschmidt and Sugden, 2013).

2.2.5. Drugs, Prevention and Control of EBV

The United States Centers for Disease Control and Prevention (CDC) notes that EBV is one of the most common human viruses found all over the world (FNIL, 2016). Most people get infected with EBV at some point in their lives. On its part, the Center for the Biology of Chronic Disease (CBCD) states that, while malaria is not a risk for those who live in the United States and other Western nations, EBV is still a dangerous virus (FNIL, 2016).

While there is no EBV vaccine, the CBCD recommends that in light of the medical evidence, infected individuals take Gene-Eden-VIR or Novirin (FNIL, 2016). The formula of these natural, antiviral supplements was designed to help the immune system target the latent EBV (FNIL, 2016). Studies showed that the Novirin formula is effective against the herpes family of viruses and EBV is a member of the herpes family (FNIL, 2016).

2.3. Epstein-Barr virus and Malaria Co-infection

Epstein-Barr virus and malaria are two infections that can each be controlled on their own, but a new study in PLOSPathogens Journal shows that co-infection can perhaps become more lethal than each infection alone, providing one possible explanation for why young children are so much more vulnerable to severe malaria (Berrin, 2015). Epstein-Barr virus may contribute to the development of severe malaria and malaria-related deaths in sub-Saharan African children (Berrin, 2015). Almost all African children will be infected with both Epstein-Barr virus and malaria by the age of 6-12 months. Because both of these infections occur around the same time

in these children, many are at a high risk for co-infection before the age of 1 (Berrin, 2015). Malaria and gammaherpesvirus co-infection in mice was more lethal than infection with either disease alone (Berrin, 2015). Mice recently infected with a gammaherpesvirus have a compromised immune response and that subsequent infection with *Plasmodium* parasites often leads to severe malarial anemia, and even death. The relative timing of the infections was integral to the development of severe malaria (Berrin, 2015).

Acute EBV infection suppresses the humoral immune response, which works through the production of antibodies. The humoral immune response is also important in fighting malaria (Berrin, 2015). When a *Plasmodium* infection occurs during an acute gammaherpesvirus infection, the gammaherpesvirus weakens the immune response to *Plasmodium* and the mouse is likely to develop severe malaria (Berrin, 2015). Also, a specific viral protein called M2 was essential for suppressing the immune response to the *Plasmodium* infection in the mice. M2 is speculated to be involved in preventing the cells that create the antibodies from doing their job (Berrin, 2015). In co-infected mice, M2 was essential for suppressing the immune response and leading to a severe malarial infection (Berrin, 2015).

Infectious disease researchers now believe that a co-infection with malaria parasite and the EBV can increase the mortality of malaria patients (FNIL, 2016). According to a study, "Malaria and Epstein-Barr virus: A Lethal Combination", which was published in PLoS Pathogens Journal, this could explain why young children are susceptible to severe malaria. Children in sub-Saharan Africa become infected by Epstein-Barr virus in infancy and it is quite difficult to distinguish from other mild childhood illnesses (FNIL, 2016). According to the authors, almost all African children will be infected with both Epstein-Barr virus and malaria by the age of 6-12 months.

Because both of these infections occur around the same time in these children, many are at a high risk for co-infection before the age of 1 (FNIL, 2016).

Also, co-senior author of the study, Tracey Lamb(PhD) an assistant professor of pediatrics at Emory University School of Medicine in Atlanta, Georgia, USA, reported that EBV can produce weeks-long suppression of the immune system. Their tests with mice show that infection with a gammaherpesvirus compromises the animal's immune response and subsequent infection with Plasmodium parasites often leads to severe malarial anemia, and even death (FNIL, 2016). Their study further suggests that malaria complications often leading to deaths may be partially due to EBV infection (FNIL, 2016). Transmission of EBV takes place early in life and is associated with human contact through saliva and BL affects mainly children with boys being more susceptible than girls (Orem *etal*., 2007).

In Uganda, children with higher baseline titers to EBV antigens are at a higher risk of developing BL, and higher antibody levels were detected many years before BL diagnosis (Orem *etal*., 2007). The capacity of EBV to stimulate B-cell proliferation inducing malignant phenotype strongly supports an etiological role of EBV in BL (Orem *etal*., 2007). In 1997, the International Agency for Research on Cancer concluded that there was sufficient evidence for the carcinogenicity of EBV in the causation of BL (Orem *etal*., 2007). Although *P*. *falciparum* is not considered as an oncogenic agent, its role in the development of BL has been suggested by the shared geographic distribution of eBL and holoendemic malaria (Orem *etal*., 2007). Both diseases show highest incidence rates in the lymphoma belt or malaria belt, an area around the equatorial Africa (+/-10º) where average temperature exceeds 15.5ºC and have yearly rainfall higher than 50 ml (Orem *etal*., 2007).

Data on associations between the sickle-cell trait (HbAS) and the multiplicity of infection, the number of genetically distinct parasites causing an infection, are limited and results have been conflicting (Mockenhaupt, *et al.,* 2004). A potential reason for these discrepancies is that, depending on the epidemiological context, high multiplicity of infection may reflect either lack of protection against infection, allowing the establishment of a larger number of patent parasites, or protection against symptomatic disease, allowing parasite clones to "stack up" since patients are less likely to seek care and receive antimalarial therapy (Gong *et al.,* 2012). Children living in malaria-endemic areas have elevated Epstein-Barr Virus (EBV) loads in the circulation and acute malaria infection leads to increased levels of circulating EBV that are cleared after anti-malaria treatment (Akin *et al.,* 2013). Infectious disease researchers at Emory have reported that their experiments with mice show that co-infection with a virus closely related to EBV can make a survivable malaria parasite infection lethal (Emory University, 2015).

Detection of insecticide resistance should be an essential component of all national malaria control efforts to ensure that the most effective vector control methods are being used. The choice of insecticide for IRS should always be informed by recent, local data on the susceptibility of target vectors (WHO, 2015).

2.4. Sickle Cell Disease

Sickle-cell disease or sickle cell anaemia is an autosomal recessive disease caused by haemoglobin S, an oxygen-carrying protein in blood cells. A single point mutation in the nucleobase sequence of chromosome 11 causes the sixth amino acid in the haemoglobin protein, glutamic acid, to be replaced by valine, changing standard haemoglobin beta into haemoglobin S.(National Institute of Health, 2009). The sickle shape is caused by haemoglobin's resulting structural change, and it is from this structure that the disease gets its name.(National Institute of

Health, 2012). The sickle-cell anaemia is also a blood disease in which red blood cells reveal an abnormal crescent (or sickle) shape when observed under a conventional microscope (Instituto Gulbenkian de Ciencia, 2011). It is an inherited disorder, the first ever (1949) to be attributed to a specific genetic modification (mutation) by Linus Pauling, a two-time Nobel laureate.

The cause of sickle-cell anaemia was attributed to a single base substitution in the DNA sequence of the gene encoding the beta chain of haemoglobin, the protein that carries oxygen in red blood cells (Instituto Gulbenkian de Ciencia, 2011).Only those individuals who inherit two copies of the sickle mutation (one from their mother and the other from their father) develop sickle cell anaemia. If untreated, these individuals have a shorter than normal life expectancy and as such it would be expected that this mutation would be rare in human populations. This is however, far from being the case (Instituto Gulbenkian de Ciencia, 2011). Observations made during the mid-20th century and building on Pauling's findings, revealed that the sickle mutation is, in fact, highly selected in populations from areas of the world where malaria is very frequent, with sometimes 10-40% of the population carrying this mutation (Instituto Gulbenkian de Ciencia, 2011).

Individuals carrying just one copy of the sickle mutation (inherited from either the father or mother) were known not to develop sickle cell anaemia, leading rather normal lives. Sickle-cell disease is one of the most common severe monogenic disorders in the world. Haemoglobin polymerisation, leading to erythrocyte rigidity and vaso-occlusion, is central to the pathophysiology of this disease, although the importance of chronic anaemia, haemolysis, and vasculopathy has been established (Reese *et al.*, 2010).The sickle haemoglobin (HbS) is a structural variant of normal adult haemoglobin (HbAA). Adult haemoglobin (HbAA) is made up of two alpha and two beta globin chains. HbS is the result of a single point mutation

(Glu → Val) on the sixth codon of the beta globin gene (Ingram, 1959). Homozygotes for haemoglobin S (HbSS) with two affected beta chains develop sickle cell disease, in which polymerized haemoglobin causes red blood cells to sickle and occlude blood vessels. Vaso-occlusion affects many organs and tissues, and results in high morbidity and mortality. Heterozygotes for sickle haemoglobin (HbAS) have sickle cell trait and are generally asymptomatic (Bunn, 1997).

Clinical management is basic and few treatments have a robust evidence base. One of the main problems of sickle-cell disease in children is the development of cerebrovascular disease and cognitive impairment, and the role of blood transfusion and hydroxycarbamide for prevention of these complications is starting to be understood (Reese *et al.,* 2010).

Recurrent episodes of vaso-occlusion and inflammation result in progressive damage to most organs, including the brain, kidneys, lungs, bones, and cardiovascular system, which become apparent with increasing age (Reese *et al.,* 2010). Most people with sickle-cell disease live in Africa, where little is known about this disease; however, it is known that the disorder follows a more severe clinical course in Africa than for the rest of the world and that infectious disease have a role in causing this increased severity of sickle-cell disease (Reese *et al.,* 2010).Despite the disease's lethal symptoms, it protects the carrier from malaria, which is why sickle cell alleles are most common in people of African descent (about 7% of people of African descent carry an allele), and other areas where malaria is prevalent (Ashley-Koch*et al.,* 2000). Sickle cell trait is hypothesized to have evolved because of the vital protection from malaria it provides (Taylor *et al.,* 2013).

Furthermore, individuals with HbAS tend to survive better than individuals with HbSS as they are not exposed to the same severity of risks yet still receive protection from malaria (Ashley-

Koch*et al.,* 2000). Individuals have three microRNAs (miR-223, miR-451, let-7i) that are effective in reducing *P. falciparum* growth and replication, and the latter two are increased in HbAS and HbSS individuals when compared to HbAA individuals (Taylor *et al.,* 2013). The microRNAs are transformed into the parasite and are inserted into its mRNA in a method similar to trans-splicing or splice-leader trans-splicing, inhibiting translation and reducing P. falciparum growth (Taylor *et al.,* 2013). Therefore, HbAS and HbSS individuals have a genetic advantage over HbAA individuals (Taylor *et al.,* 2013).

Sickle-cell disorder (SCD) usually manifests early in childhood and affects millions throughout the world. It occurs more commonly in people or their descendants from parts of tropical and sub-tropical regions where malaria is or was common (Ilesanmi, 2013). It occurs primarily in those of African descent, but it also afflicts people of Mediterranean, Middle Eastern and Asian origins (Ilesanmi, 2013).

Nigeria bears the highest burden of SCD in sub-Saharan Africa (Ilesanmi, 2013). The nation has the largest burden of sickle disorder in the whole world (Ilesanmi, 2013). Carriers of the sickle cell gene (HbAS) have over the past centuries flourished and multiplied in tropical sub-Saharan Africa because their carrier status protected them from succumbing to the deadly falciparum malaria prevalent in the region (Ilesanmi, 2013). Also, the nation has the largest population of carriers (Ilesanmi, 2013). About 2% of babies born to Nigerian women have sickle cell anemia, i.e., about 100,000 babies are born annually with sickle cell anemia (Ilesanmi, 2013).

It is widely accepted that sickle cell trait protects the carrier from malaria because only a small number of *P. falciparum* are able to infect sickled cells due to their shapes and increased rates of phagocytosis of infected sickled cells (Lamonte *et al.,* 2012). Both males and females have an equal chance of getting sickle cell as it is not a sex-linked disease. Nitric oxide, a substance that

helps blood vessels dilate, is up to two times more available in women than in men with the sickle cell anemia genetic condition (American Heart Association, 2002).

CHAPTER THREE

3.0 MATERIALS AND METHOD

3.1 Study Area

The study was carried out in Zaria, Kaduna State, Nigeria. Zaria is a major city in Kaduna State in the Northern Nigeria and is about 100km from the Atlantic Ocean. It is located on the coordinates 11.04^{o}N, 7.42^{o}E/ 11.067^{o}E. The population of Zaria is 695,089 (NPC, 2006). It is characterized by a tropical climate with two seasons; a rainy season of approximately 210 days (March to September), with a mean annual rainfall of 1092.8mm, and the dry season which spans between October and February. The monthly mean temperature shows a maximum of 39.7^{o}C in June and 13.8^{o}C in December (Ukegbu, 2005).

3.2 Study Design

This study was a cross-sectional studywhich included the use of administered questionnaires and laboratory analysis of blood samples collected from patients who agreed to participate in the study. A total of 279 consenting patients were recruited for the study at the general and haematology laboratories of Ahmadu Bello University Teaching Hospital (ABUTH), Zaria.

3.3 Study Population

The study population comprised of 125 (44.8%) consenting male and 154 (55.2%) femalepatients within the age-range of 0-70 years who reported to ABUTH, Zaria presenting with fever and had temperatures above 38^{o}C (febrile group; 58.8%: 164/279), patients with allsorts of cancer (cancer group; 27.2%: 76/279) and thoseconfirmed by the Haematology Unit of

the hospital to be sickle-cell patients(sickle-cell group; 14.0%: 39/279). The hospital doctors and laboratory staff were instrumental in ascertaining the clinical conditions of the sampled patients.

3.4 Ethical Consideration and Consent

Ethical approval was obtained from the Ethical Committee of the ABUTH Shika-Zaria (Appendix I). Both verbal and written consent (Appendix II) were obtained from the patients before they were recruited into the study. Consent of underaged patients were obtained from their parents/guardians while those who were illiterate had the consent form read and interpreted tothem.

3.5 Inclusion and Exclusion Criteria

The inclusion criteria included all male and female febrile, cancer and sickle-cell patients within 0-70 yearswho consented to participate in the study, while the exclusion criteria included all patients who did not consent, had illnesses other than fever, cancer or sickle-cell disease.

3.6 Sample Size Calculation

The sample size was calculated using the formula below:

$$n = \frac{PQ}{(E/Z)^2}$$

Where;

Z= 1.96 for 95% confidence interval

P= prevalence of 23.8% obtained in a previous studyon EBV-related malignancies in Kano, Nigeria(Jain, *et al.*, 2011).

Q= 100-P= 100-23.8= 76.2.

E= Allowable error = 5%

n= number of samples to be collected.

n= 23.8 x 76.2/ $(5/1.96)^2$= 279.0

The sample size calculated was 279 hence,279 samples were collected and used for the study.

3.7 Data Collection using Questionnaire

Structured questionnaires (Appendix III)were used to collect information on patient's sociodemographic data**,** clinical data and some risk factors that might be associated with EBV-related cancers and co-infectionwith EBV and *Plasmodium* species among the selected patients.

3.8 Sample Collection

Five mililiters(5mls) of blood was collectedfrom the 279 consenting patients with the assistance of the hospital laboratory staff via venipuncture and shared into plain tubes (for serology) and EDTA tubes (for thin and thick blood films) between themonths of October, 2015 and April, 2016, at the ABUTH, Shika-Zaria. Two milliliters (2ml) of the blood obtained in the EDTA tubes was immediately used for thick and thin blood smears on clean, grease-free slidesfor malaria parasite detectionby light microscopy while thatobtained in the plain tubes was centrifuged at 3000rpm for 5minutes in order to separate the serum from the blood cells. The serum thus obtained was preserved at -15^{o}C in the Haematology Unit of ABUTH, Zaria, until all the samples were collected and ready for the serological analysis. The serum samples were then transported in ice-packsto the Virology/Immunology laboratory in the Department of Microbiology, Faculty of Life Sciences, Ahmadu Bello University Zaria, where EBV detection and patients' ISRsto EBVNA-1were determined.

3.9 Sample Analysis

The prepared blood filmswere analysed for both the presence and density of *Plasmodium* species using microscopy and the atlas of Medical Parasitology. Also, analysis for the presence of IgG to EBVNA-1-1 and determination of patients' ISRs to EBVNA-1-1 were carried out using ELISA.

3.9.1 Microscopy for detection of *Plasmodium*species and their density/µL of blood

Thin and thick blood smears were prepared for each of the samples. Films obtained were used to determine the presence of *Plasmodium*species and their density/µL of blood among the selected patients attending ABUTH, Zaria, using light microscope and the Atlas of Medical Parasitology (Chiodini *et al.*, 2003) as described below.

3.9.1.1 Microscopy

Completely clean and grease-free microscope slides were obtained and a small drop of blood by means of an edge of another glass slide was placed at the center of one slide while a larger drop was added to the center of another glass slide (i.e., former for thin blood smear while the latter for thick blood smear).Without delay, the thin film was made by spreading the drop of blood using the smooth edge of another glass slide, while the thick blood smear was made by spreading out the large drop of blood to cover an even area.The smears were then placed horizontally in a slide holder and allowed to air-dry in a safe place (free from dust and insects) after which they were labeled for easy identification(Cheesbrough, 2009).

Furthermore, the thin film slides were placed on a staining rack and fixed with absolute methanol for 2-3 minutes. The thick blood smears were not fixed, to allow lysis of the red blood cells.Both the thick and thin smears were stained with Giemsa stain by placing the slides on a staining rack and flooding the smears with sufficient amount of the Giemsa stain for 25-30 minutes.The excess

stain was washed off with clean water while the back of the slides were wiped clean with tissue paper towel. The slides were then placed vertically in a draining rack and left to air-dry(Cheesbrough, 2009). When the stained smears were completely dry, a drop of immersion oil was applied to the smears and the smears were viewed under the microscope for *Plasmodium* spp. employing the atlas of Parasitology(Chiodini *et al.*, 2003) as a useful help in identifying the *Plasmodium* species(Cheesbrough, 2009).

3.9.1.2Estimating Malaria Parasite Density

The parasite density was estimated by multiplying the average number of parasites observed per high power field (100x objective and 10x eyepiece) by 500.The factor of 500 was proposed by Greenwood and Armstrong(Cheesbrough, 2009). They reported this method to be more accurate and quicker than counting the parasites against white cells as in the other methods (Cheesbrough, 2009).Between10-50 fields were examined, depending on parasitaemia, to determine the average number of trophozoites per high power field (HPF). Ten fields were recommended to be sufficient when the parasite density is high (Cheesbrough, 2009).

3.9.2 Serology for detection and quantification of anti-EBVNA-1

The stored serum samples were thawedat 25-28^{o}C by placing the tubes on the laboratory bench. The serum samples were then assayed for IgG antibodies to EBVNA-1-1 usingthe Epstein-Barr virus Nuclear Antigen-1 (EBVNA-1-1)(IgG)ELISA test kits (Diagnostic Automation, Inc., USA, 2015)to qualitatively detect and further computethe ISRs ofthe selected patients attending ABUTH, Zaria to EBVNA-1-1. The ISR for each specimen was computed by dividing the specimen's optical density value by the cutoff calibrator value. The kit has a specificity of 100% and a sensitivity of 97.8% (Diagnostic Automation, Inc., USA, 2015).

3.9.2.1 Principle of the ELISA

The Enzyme-Linked Immunosorbent Assay (ELISA) used in this study relies on the ability of biological materials (i.e., EBVNA-1) to adsorb to plastic surfaces such as polystyrene (solid phase). When the EBVNA-1bound to the solid phase are brought into contact with a patient's serum, antibodiesspecific for the antigen, if present, will bind to the antigen on the solid phase forming antigen-antibody complexes. Excessantibody is removed by washing. This is followed by the addition of goat antihuman IgG conjugated with horseradish peroxidase which then binds to the antibody-antigen complexes. The excess conjugate is removed by washing followed by the addition of chromogen/substrate (tetramethylbenzidine, TMB). If specific antibody to the antigen is present in the patient's serum, a blue colour develops. When the enzymatic reaction is stopped with 1N H_2SO_4, the contents of the well turn yellow. The colour, which is indicative of concentration of antibody in the serum, can be read on a suitable spectrophotometer or ELISA microwell plate reader (Diagnostic Automation, Inc., USA, 2015).

3.9.2.2 The Assay Procedure

The assay was carried out according to the manufacturers' instructions. All reagents and samples were brought out from the refrigerator and allowed to come to room temperature (25-28^{o}C) before use. The patients' serum were diluted in the ratio 1:21 with the sample diluent provided. About 100µL of the appropriately diluted calibrator, controls and patients' serum were added to individual wells. The set-up was incubated at room temperature (25-28^{o}C) for 25 minutes.

At the end of the incubation period, all liquid in the wells were shaken out to wash out excess antibody in the set-up above. The wash buffer (50ml of the wash buffer was made up to 1L with distilled water) was used to wash repeatedly four times. After the final wash, the ELISA plate was blot on paper towel to remove all liquids from the wells.Subsequently,100µL of

theConjugate was added to eachwell, including the blank well. The set-up was then incubated again at room temperature for 25 minutes. Washing was repeated as done above.Thereafter, a further100µL of the Chromogen/Substrate Solution(TMB)was added to each well and incubatedagain at room temperature for15 minutes. The reaction was then stopped by the addition of 100µL of the Stop Solution (1N H_2SO_4) to each well.The developed colour was read on an ELISA plate reader equipped with a 450nm filter.

3.9.2.3 Interpretation of Results

The Immune Status Ratio (ISR) for each specimen was determined by dividing the specimen optical density (O.D.) value obtained from the ELISA assay by the cutoff calibrator value. The cutoff calibrator value was determined by multiplying the mean O.D. for the calibrator by the correction factor.The Patients' ISR values were interpreted either as lowpositive (≤0.90) or highpositive (≥1.10).High ISR values are a risk factor for EBV-related cancers.

3.9.3 Data Analysis

The data obtained using questionnaires and results were analysed using SPSS version 17.0 statistical software. ANOVA, Odd ratio and Chi-square (χ^2) were also used to test the association between variables. Statistical significance was at $P<0.05$ and 95% confidence level.

CHAPTER FOUR

4.0 RESULTS

Results obtained in this study showed*Plasmodium falciparum* as the only *Plasmodium* species detected among the study population (Table 4.1).An overall malaria prevalence of 63.4%

(177/279)was found in the study. *Plasmodium falciparum*malariamostly occured among the cancer group (89.5%) but was least among the febrile group (48.2%). There was significant statistical association between malaria infection and the study group of the patients (P=0.002, df=2, χ^2=49.10)as shown in Table 4.2.

The parasite densityamong the different study groups is shown in Table 4.3.The parasite density was reported as low (3,500-10,000 parasites/μL of blood), moderate (11,000-20,000 parasites/μL of blood) or high (≥25,000parasites/μL of blood). Among the study population 26.5% (74/279) had low parasitaemia while 21.5% (60/279) had high parasitaemia. Using ANOVA to compare the mean values, no statistical differencewas found between the study groupof patients and their having low (F=0.063)or moderate (F=0.121) parasitaemia.However, there was significant differencebetween study group and high parasite densityamong the study population (F=0.02, P=0.113, df=2, χ^2=29.06).

An overall EBV prevalence of 100% was found amongpatients inthis study, indicating a 63.4% co-infection of EBV and *Plasmodiumfalciparum* among the study population.

Table 4.1: Prevalence of *Plasmodium* species among the selected patients attending ABUTH, Zaria.

Plasmodium species	Prevalence of *Plasmodium*species (%) (n=279)
P. falciparum	177 (63.4)

P. malariae	0	(0)
P. ovale	0	(0)
P. vivax	0	(0)

Table 4.2: Distribution of *P. falciparum* among the selected patients attending ABUTH, Zaria.

Study Group	Number Examined	Number Positive (%)	χ^2	P-value	df
Febrile	164	79 (48.2)	49.10	0.002	2

Cancer	76	68 (89.5)
Sickle-cell	39	30 (76.9)
Total	279	177 (63.4)

Table 4.3: The*P. falciparum* density among the selected patients attending ABUTH, Zaria.

Study Group	Number Examined	Parasite Density		
		*Low (%)	**Moderate (%)	***High (%)
Febrile	164	35 (21.3)	17 (10.4)	27 (16.5)

Cancer	76	18 (23.7)	20 (26.3)	30 (39.5)
Sickle-cell	39	21 (53.9)	6 (15.4)	3 (7.7)
Total	279	74 (26.5)	43 (15.4)	60 (21.5)

*F=0.063; **F=0.121; ***F=0.02; df=2; P=0.113; χ^2=29.06

Key:

Low: 3,500-10,000 parasites/µL of blood.

Moderate: 11,000-20,000 parasites/µL of blood.

High: ≥ 25,000 parasites/µL of blood.

Themean immune status ratio (ISR) of the study population is shown in Table 4.4.The highest mean ISR (1.34) was found among patients who had both cancer and malaria (68/279)while the least mean ISR (0.59) was found among febrile individuals who did not have malaria(85/279). There was statistical association between infection with malaria and EBV-associated cancers among the study population (χ^2=21.01, P=0.0001, OR=7.08, F=0.040, df=2, CI=95%).Table 4.5

shows the ISRand corresponding *P. falciparum* density among the study population. The highest parasite density (≥ 25,000 parasites/µL of blood) corresponded to an equally higher ISR level (1.97) while the least parasite density (3,500-10,000 parasites/µL of blood) corresponded to a lower ISR (0.91). A statistical association was found between the parasite density and the mean ISR of the study population (χ^2=18.01, P=0.001,df=3).

The*P. falciparum* density in relation to sex of the study population is shown in Table4.6. A higher parasitaemia was found among the female sex (67.5%: 104/279) than among the male sex (58.40%: 73/279). Further statistical analysis show no association between the male sex of the study population and malaria parasite density (F=0.122) while an association was found between the female sex and malaria parasite density (F=0.043; df=1, χ^2=27.56, P=0.762).

Table 4.7 shows the relationship between the place of residence and parasitaemia among the study population. About 63.08% (176/279) of the study population were from Kaduna state, the study location, while 36.91% (103/279) of them were from states in the neighborhood of the study area. However, those from Kaduna had higher malaria cases (58.0%: 102/176) than their counterparts from outside Kaduna state (72.8%: 75/103). Using ANOVA,the results showed no statistical difference between residence in Kaduna state and malaria infection (F=0.181) while statistical differenceexisted between residenceoutside Kaduna state and malaria infection (F=0.050, χ^2=6.11, df=1, P=0.451).

There was statistical association between age-group and malaria infection among the selected patients as shown in Table 4.8. The distribution of *P. falciparum* by the age-group of the study population showed that the 61-70 age-group had the most parasitaemia (100%) while the least parasitaemia (52.8%) was among the 21-30 age-group (χ^2=78.96, df=6, P=0.004, F=0.01).

Table 4.9 shows the type of occupation and*P. falciparum* distribution among the study population. The highest case of malaria was found among farmers (73.3%: 11/15) while the least was among the artisans (44.8%: 13/29). Statistical analysis shows asignificant association between type of occupation and infection with malaria (χ^2=67.08, df=5, P=0.017).

The level of education and distribution of malaria parasitaemia among the selected patients attending ABUTH, Zaria is as shown in Table 4.10in which the highest case of malaria was found among those who have had only primary education as at the time of conducting this research (87.5%: 56/64). The least parasitaemia was found among those who have had a tertiary level education (41.2%: 39/94). There was statistical association between the measured variables (χ^2=21.71, df=3, P=0.002).

Table 4.4: The Immune Status Ratio of the selected patients attending ABUTH, Zaria.

Study Group	Malaria status	Number Examined	Group ISR	Mean ISR
	Malaria Positive	79	99.54	1.26
Febrile	Malaria Negative	85	50.15	0.59

	Malaria Positive	68	101.84	1.34
Cancer	Malaria Negative	8	7.76	0.97
	Malaria Positive	30	48.75	1.25
Sickle-cell	Malaria Negative	9	5.94	0.66

ISR=Immune status ratio. F=0.040; df=2; OR=7.08; CI=95%; χ^2=21.01; P=0.0001

Key:
Low positive: ≤ 0.90/10µL of serum; High positive: ≥1.10/10µL of serum

Table 4.5: The Immune Status Ratio and *P. falciparum* density among the selected patients attending ABUTH, Zaria.

Parasite Density	Number Examined	Group ISR	Mean ISR
Nil	102	63.85	0.63

Low	74	67.11	0.91
Moderate	43	64.70	1.50
High	60	118.32	1.97

ISR=Immune status ratio χ^2 = 18.01; df=3; P=0.0013

Key:
Low positive: ≤ 0.90/10µL of serum; High positive: ≥1.10/10µL of serum:

Low: 3,500-10,000 parasites/µL of blood.

Moderate: 11,000-20,000 parasites/µL of blood.

High: ≥ 25,000 parasites/µL of blood.

Table 4.6: The *P. falciparum* density in relation to sex of the selected patients attending ABUTH, Zaria.

Sex	Number Examined	Number Positive (%)	Parasite Density		
			Low (%)	Moderate (%)	High (%)
*Male	125	73 (58.4)	29 (23.2)	16 (12.8)	18 (14.4)

**Female	154	104 (67.5)	45 (29.2)	27 (17.5)	32 (20.8)
Total	279	177 (63.4)	74 (26.5)	43 (15.4)	60 (21.5)

*F=0.122; **F=0.043; df=1; χ^2=27.56; P=0.762

Key:

Low: 3,500-10,000 parasites/µL of blood.

Moderate: 11,000-20,000 parasites/µL of blood.

High: ≥ 25,000 parasites/µL of blood.

Table 4.7: Place of residence and distribution of *P. falciparum* among the selected patients attending ABUTH, Zaria.

Place of Residence	Sex	Number Examined	Number Positive	% Positive
	Male	82	47	57.3

*Kaduna State	Female	94	55	58.5
	Total	176	102	58.0
	Male	43	26	60.5
**Outside Kaduna	Female	60	49	81.7
State	Total	103	75	72.8

*F=0.181; **F=0.05; df=1; χ^2=6.11; P=0.451

Table 4.8: Distribution by age-group of *P. falciparum* among the selected patients attending ABUTH, Zaria.

Age-group	Number Examined	Number of Males Positive (%)	Number of Females Positive (%)	Overall Positive cases (%)
0-10	34	11 (32.4)	12 (35.3)	23 (67.6)

11-20	44	14 (31.9)	19 (43.2)	33 (75.0)
21-30	53	13 (24.5)	15 (28.3)	28 (52.8)
31-40	59	15 (25.4)	19 (32.2)	34 (57.6)
41-50	44	6 (13.6)	18 (40.9)	24 (54.6)
51-60	41	11 (26.8)	20 (48.8)	31 (75.6)
61-70	4	3 (75.0)	1 (25.0)	4 (100.0)
Total	279	73 (26.2)	104 (37.3)	177 (63.4)

χ^2=78.96; P=0.044; F=0.011; df=6;

Table 4.9: Occupation and *P. falciparum* distribution among the selected patients attending ABUTH, Zaria.

Occupation	Number Examined	Number Positive	% Positive

Civil Service	54	27	50.0
Artisan	29	13	44.8
Business	32	19	59.4
Farming	15	11	73.3
Pensioner	12	7	58.3
No formal Occupation	137	100	73.0
Total	279	177	63.4

χ^2 = 67.08; df=5; P=0.017

Table 4.10: Level of Education and *P. falciparum* distribution among the selected patients attending ABUTH, Zaria.

Educational Level	Number Examined	Number Positive	% Positive

No formal Education	58	38	65.5
Primary	64	56	87.5
Secondary	63	44	69.8
Tertiary	94	39	41.2
Total	279	177	63.4

χ^2 = 21.71; df=3; P=0.002

There was a significant statistical association between type of cancer and distribution of *P. falciparum* among the study population as shown in Table 4.11. The cancer types with the highest distribution of malaria parasitaemia were the head and neck cancers 18 (100.0%: 18/18)

and Lung cancer (100.0%: 4/4) while the least parasitaemia was found among those with cervical cancer (81.8%: 9/11) (P=0.0486, df=6, χ^2=19.01).

Some likely risk factors established by literature to predispose to EBV infection were evaluated among this study population and presented in Table 4.12-4.14. The risk factors with the highest frequency and statistical association among patients were exposure to X-rays (59.9%: 167/279), a family history of cancer (15.4%: 43/279)and the use of oral contraceptives (14.7%: 41/279). The use of bleaching creams had the least occurrence (3.2%: 9/279) with no statistical significance (Table 4.13a).Further evaluation of the genotype as a risk factor for *P. falciparum* infection is shown in Table 4.13b. The highest case of malaria was found among the sickle-cell carrier variant genotype, HbAC (100.0%: 4/4) while the least parasitaemia was found among the sickle-cell carrier genotype, HbAS (76.9%: 30/39). No significant statistical association was found between the genotype of the patients and *P. falciparum* malaria infection (P=0.093, df=3, χ^2=53.91).

The ABO blood type as a risk factor for *P. falciparum* infection among the study population was evaluated and presented in Table 4.13c.The highest *P. falciparum* infection was found among the blood group O (77.8%: 48/141) while the least parasitaemia occurred among those with blood group B (69.1%: 38/141). No statistical association was found between the evaluated variables (χ^2=29.60, df=3, P=0.151).

Table 4.11: The distribution of *P. falciparum* among the selected cancer patients attending ABUTH, Zaria.

Cancer Type	Number Examined	Number Positive	% Positive

Prostate	2	1	50.0
Breast	21	19	90.5
Intestinal	6	5	83.3
Lung	4	4	100.0
Head and Neck	18	18	100.0
Skin	14	12	87.7
Cervical	11	9	81.8

χ^2 = 19.01; df=6; p=0.0486

Table 4.12: Likely Risk Factors Predisposing to cancer among the selected patientsattending ABUTH, Zaria.

Risk Factor	Number Examined	Response	χ^2	df	P-value

		YES (%)	NO (%)			
Use of oral Contraceptives	279	41 (14.7)	238 (85.3)	1.505	1	0.681
Smoking	279	11 (3.9)	268 (96.1)	1.318	1	0.725
Alcohol consumption	279	13 (4.7)	266 (95.3)	3.192	1	0.993
Use of bleaching creams	279	9 (3.2)	270 (96.8)	0.090	1	0.993
Family History of Cancer	279	43 (15.4)	235 (84.2)	12.330	1	0.009
Exposure to X-rays	279	167 (59.9)	112 (40.1)	21.844	1	0.004

KEY: P≤0.05= Significant

Table 4.13: The genotype and *P. falciparum* infection among the selected patients attending ABUTH, Zaria.

Genotype	Number Examined	Number Positive	% Positive

HbAA	63	59	93.7
HbAS	50	44	88.0
HbSS	39	30	76.9
HbAC	4	4	100.0
Total	156	137	87.8

χ^2 = 53.91; df=3; P=0.093

Key:

HbAA= normal genotype

HbAS= sickle-cell carrier genotype

HbSS= sickle-cell genotype

HbAC= a variant of the AS genotype

Table 4.14: The ABO blood type and *P. falciparum* infection among the selected patients attending ABUTH, Zaria.

ABO blood type	Number Examined	Number Positive	% Positive

A	59	42	71.2
B	55	38	69.1
AB	17	13	76.5
O	63	49	77.8
Total	194	142	73.2

χ^2 = 29.60; df=3; P=0.151

CHAPTER FIVE

5.0 DISCUSSION

In this study, only a single species of *Plasmodium* (*P. falciparum*) was found among the selected patients attending ABUTH, Zaria. This finding suggests a successful adaptation of the *Plasmodium falciparum* to the study area and the African climate at large. Absence of the other species (*P.malariae*, *P.ovale* and *P.vivax*) reflects a possibility of their failure to adapt, infect and complete their life cycles within the study area, leading to their gradual extinction. Within the same region as this study area (Sokoto state-Nigeria) results of Udomah*etal.* (2015) also indicated that *Plasmodiumfalciparum* was responsible for all the cases of malaria parasitaemia found among theirstudy subjects.In Bayelsa-Nigeria, Abah and Temple (2015) reported that *P. falciparium* was the only species observed in their study population.Similarly, Ayodele *et al.* (2014) found that all their study subjects were infected with only one species of *Plasmodium* (*P. falciparum*) as reported in this study.

In contrast, findings of Andargachew *et al.* (2013) and that in Abuja-Nigeria(Nmadu *et al.,* 2015)where children investigated for malaria were found to be dominantly infected with *P. falciparum*, indicated that other species were present but at lower prevalence and frequency of detection than the *P. falciparum*species. A majority of infections in Africa are caused by *Plasmodiumfalciparum*, the most dangerous of the four human malaria parasites (Abah and Temple, 2015; Wassrmer *et al.,* 2015).*Plasmodium falciparum* is the most prevalent malaria parasite on the African continent. It is responsible for most malaria-related deaths globally (WHO, 2015).

The prevalence of *P. falciparum* malaria among the selected patients attending ABUTH, Zaria, was 63.4% while the prevalence of EBV was 100%. The co-infection of EBV and *Plasmodiumfalciparum*was 63.4%. Themalaria prevalence found in this study is lower thanthe 80.5% reported by Olasehinde *etal*. (2010) in Ota-Nigeria and the 90.4% malaria prevalence

reported in Abia State-Nigeria by Kalu, *et al.,* in 2012. In Sokoto State-Nigeria, Udomah*etal.* (2015) reported a prevalence of 52.2%. These findings show the magnitude of the malaria burden prevalent in the study area, Nigeria, as well as in holoendemic equatorial Africa in which *P. falciparum* infection occurs throughout the year (Orem *etal.,* 2007; Brooks *et al.,* 2010). In southern Nigeria, Abah and Temple (2015) reported a malaria prevalence of 63.3% among their Bayelsa state study population, similar to the prevalence obtained in this study.

The findings of Abah and Temple (2015) were attributed to their environment which apart from being typical of the mangrove vegetation and tropical rain forest zone is a lower coastal plain with lots of water which encourages malaria vector breeding, since epidemiological patterns of malaria infection are governed by environmental parameters that regulate malaria vector populations. However, in the Northern part of Nigeria, where this study was conducted, the persistently higher temperatures are attributable to the reported high malaria prevalence, as people preferred to sleep bare-bodied, outside insecticide treated bed nets, thereby exposing themselves to the malaria vector.

Again, this study reported a parasite density of above 25,000 parasites/μL of blood. In Southern Ethiopia however, malaria parasite density of less than 25,000 parasites/μL of blood, between 25,000-50,000 parasites/μLof blood and greater than 50,000 parasites/μLof blood have been reported, agreeing with the findings in this study. This emphasizes the malaria burden in sub-Saharan Africa.Malaria still kills more people than HIV/AIDS or any other killer disease and it is endemic throughout Nigeria (Oyewole *et al.,* 2011).In 2015, 95 countries and territories had ongoing malaria transmission. WHO estimates there were 214 million cases of malaria and 438,000 deaths. Sub-Saharan Africa carries a disproportionately high share of the global malaria burden. The region was home to 88% of malaria cases and 90% of malaria deaths (WHO, 2015).

The prevalence of EBV found in this study was 100%. This prevalence was also reported by Ferrari *et al.* (2014) among their study population. The high prevalence of EBV among most human populations is not unconnected to the ease of its transmission from an infected host to a susceptible host through a simple and common route. EBV is transmitted primarily through contact with saliva (Rochford, 2009). Oral transmission through direct contact with infectious saliva is considered to be the primary route of transmission in young adults. The virus can also spread through direct contact with contaminated eating utensils, toys or fingers (Rochford, 2009). Indirect contact with infected saliva is thought to be the primary route for infection among infants and young children. In developing countries, premastication of food by mothers suggest a possible reason for higher rates of transmission to infants (Rochford, 2009).

The mean ISRs were higher among cancer patients who were also positive for *P. falciparum* while a low mean ISR was found among the febrile patients who were negative for malaria. Furthermore, there was significant statistical association between the mean ISR of patientsand *P. falciparum* infection. A seven-fold increased chance of a malaria-positive patient to develop EBV-related cancers more than a malaria-negative individual was found among the study population.This finding agrees with that of Stevens *et al.* (2005) in which they found that a stronger IgG reactivity to EBVNA-1 exists among patients with EBV-related cancers (nasopharyngeal carcinoma) than that seen in healthy EBV carriers. Repeated malaria infections especially in young children modulate the persistence of EBV and increase the risk for the development of EBV-associated cancers (Burkitt's lymphoma) (Moorman, *et. al.,* 2005). EBV is one of the best tumour markers yet discovered (Gulley and Tang, 2008).

According to Stevens *et al.* (2005) EBV DNA is usually present at low levels in the circulation of healthy EBV seropositive carriers and that stronger IgG reactivity correspond to a

significantly higher whole-blood EBV DNA levels in the EBV-associated cancer patients.This wascorroborated by other researchers in China, Thailand, Italy and Taiwan. Jin-Ching Lin and his co-workers (2004) also found that in China, patients with active infection or EBV-related cancers tend to have high levels of EBV DNA in the cell free fraction of blood (plasma or serum), whereas in healthy carriers, the virus is restricted to the intracellular compartments of blood. They also reported that healthy people who showed presence of high concentrations of antibodies against EBV DNA and proteins later developed nasopharyngeal carcinoma.

The level of EBV DNA in the blood correlates with disease severity (Gulley and Tang 2015). Epstein Barr virus DNA levels in whole blood, plasma, serum or memory B cells spike within two weeks after primary infection with EBV and then drop to low or undetectable levels over a period of several weeks to months(Gulley and Tang 2015). The low EBV viral load in most healthy EBV-infected individuals reflect the low frequency of EBV-positive B cells in the circulation, whereas the high EBV loads observed during EBV-related diseases are a result of increased numbers of EBV infected B cells(Babcock, 1999). However, differences in detection methods, sample preparation and measurement units make comparisons of EBV viral loads from different studies difficult (Fan and Gulley, 2001). From the foregoing, the detection of Anti-EBV DNA in cell-free fraction of blood samples might be used as a laboratory marker of active EBV disease when a Real-Time PCR or another quantitative method is not available. From the foregoing, it can therefore be said that the higher ISRs to EBVNA-1 obtained in this study is a likely risk factor for EBV-related malignancies.

Furthermore, results obtained in this study show a relationship between EBV and*Plasmodiumfalciparum*, such that, as the parasite density increases, a corresponding increase in the ISRwas observed, and vice-versa.This finding contrasts with that of other studies where

Anti-EBV-VCA IgG were present equally (>99%) in both patients and control among study subjects (Hon, *et al.,* 2012). Hess *etal*. (2004) established that only about 5% of adults in western societies remain EBV uninfected, thus antibody rates reach 95% or higher among elderly individuals, agreeing withthe findings in this study. Thus, the report of other researchers suggesting that a molecular linkexist between EBV and *P. falciparum* such that the membrane of *P. falciparum* contained a cysteine-rich inter-domain region 1α (CIDR1α) membrane protein 1 which has been found to be a polyclonal B cell activator which preferentially activates the memory compartment where EBV is known to persist, could have accounted for the observed co-infection between EBV and *P. falciparum* in this study.

Since malaria is not a sex-linked disease, differences in exposureto the malaria vector among the two sexes, as well as differences in immunity to parasitic infections explains the finding in this study in which more of the femaleswere infected with*falciparum* malaria than their male counterparts.This finding is contradicted by that of Ayodele*et al.*(2014), who found higher malaria prevalence among males than infemales, among their study population in Ekiti State-Nigeria. However,Kalu *et al.* (2012) found higher infection prevalence among females than males in Abia state-Nigeria, corroborating the findings in this study.

Also, in this study, the lowerincidence of *falciparum* malaria found among the 21-30 age-group suggeststhe possibility of a stability attained by their immune systems after 1-2 decades of exposure to the prevalent malaria parasite and subsequent development of memory antibody cells against the parasites. While the higher parasitaemia found among the 61-70 age-group reflects a deteriorationof immunity typical of the aged. This finding differs from that of Kalu *et al.*(2012) who reported higher malaria infection among the 21-30 age-group of their study population,

which they attributed to the habitual exposure of youths who make up this group to incessant bites of the malaria vectorsby remaining bare-bodied especially when the weather is hot.

Furthermore, since there was no statistical association between themarital status and malaria parasitaemiaamongpatients in this study, it could be concluded that the high malaria infection found among the divorced and the low infection found among the married patientsweremerely coincidental. However, there was significant statistical association between the high parasitaemia found among farmers and the lowparasitaemia found among artisans, which reflectedthe differences in the levels of exposure to the malaria vector, as determined by an individual's work environment. The farmers were thus more exposed and more likely to be bitten by the malaria vector than bankers working in a less exposed environment.

The level of education and the distribution of malaria parasites among the selected patients attending ABUTH, Zaria showed a statistical association in this study, such that the least parasitaemia was found among those patients who have had tertiary level education, while the most parasitaemia was found among those with only a primary level education. Education thus conferredknowledge on the burden of malaria, its prevention and control as well as the need toseek proper treatment when infected, thereby reducing the vulnerability to malaria infectionamong the educated.

In this study, there was asignificant statistical association between the type of cancer and infection with *P. falciparum* among the selected patients attending ABUTH, Zaria.Ahighermalaria parasitedensity was found among patients with head and neck and breast cancers, while the least parasitaemia was found among patients with prostate cancer. Epstein-Barr virus latent infection has been linked to several malignant cancers that occur in the head and

neck regions as the oropharyngeal epithelium is the main site of the virus proliferation after infection (Jibril*et al.*, 2013). This further corroborates the findings in this study about the relationship between EBV and malaria, since patients with EBV-related cancers had higher malaria parasitaemia than participant with other cancer types not known to be linked to EBV.

Risk factors predisposing to EBV-related cancers found in this study to have significant statistical association with malaria and EBV co-infectionwere exposure to X-raysand a family history of cancer.Other risk factors such as the use of oral contraceptives and the use of bleaching creams had no such statistical significance.Also, co-infection with *falciparum* malaria and EBV was found to have no significant statistical association with the genotype of patients. However, the co-infection was higher among the sickle-cell carrier variant genotype (HbAC) and the normal genotype,followed by thatamong the sickle-cell carrier genotypewhile itwas least among the sickle-cell genotype. This showed some level of protection enjoyed by the sickle-cell trait as reported by Mulama *etal.* (2014) in Kenya, that although the HbAS protects against severe malaria and hyperparasitaemia, it is not associated with viral control or endemic Burkitt lymphoma protection.

Other studies too have suggested that, in one way or another, the sickle haemoglobin gets in the way of the *Plasmodium* parasite infecting red blood cells, thus reducing the number of parasites that actually infect the host and hence confer some protection against the disease (Instituto Gulbenkian de Ciencia, 2011).Similarly, in Oyo-Nigeria, Akhigbe *etal.* (2009) reported that dominant homozygotes (HbAA) were more susceptible to plasmodial parasite infection than the sickle heterozygotes (HbAS), while the recessive homozygotes (HbSS) were most vulnerable to malaria than the other two members of the group.However, the report that the HbSSgenotype was most vulnerable to malaria did not agree with the findings presented in this study.Despite

studies demonstrating that mice genetically engineered to produce one copy of the sickle haemoglobin similar to the sickle cell trait, do not succumb to cerebral malaria, the mechanism underlying this protective effect remained elusive (Instituto Gulbenkian de Ciencia, 2011).

Furthermore,the ABO blood type had no statistical association with EBV and *P. falciparum* co-infection in this study. However, results showed blood type O had the most parasite density followed by the AB blood type whilethe least parasitaemia was found among the B blood type. This finding agrees with that of Zerihun *etal*. (2011) in Southern Ethiopia in which the ABO blood type O had the most malaria parasitaemia while the least parasitaemia was found among blood type AB. Zerihun and his coworkers (2011) further explained that there was a selective evolutionary advantage of *P. falciparum* infection on blood group O cells compared with the other blood group types (A, B or AB) in areas where malaria is endemic.Again, Gupta and Chowdhuri, (1980) maintained that susceptibility to several infectious diseases is related to the patient's blood group. According to them, although the relationship between blood group and susceptibility to malaria has been studied by several workers, the results have been contradictory. In their own study among malaria patients (1980), Gupta and Chowdhuri reported that blood group A was the most susceptible to malaria. This contradicts the findings in this study.

Gupta and Chowdhuri, (1980) further reported that the genetic make-up of individuals may cause a considerable variation in their reaction to malarial infection and that blood groups were merely an expression of genetic constitution. They also reported that qualitative and or quantitative variation in structure and chemical composition of the receptor sites on erythrocytic membrane of the various groups may play an important role in determining susceptibility. Furthermore, Panda and his co-workers stated that malaria parasites share group A antigens and hence are better tolerated by the host's immune system (Panda*et al.*, 2012). Another variation may be

ascribed to the feeding habits of the vector species. Some people are more prone to mosquitoes bite than others. Under laboratory conditions, *Anopheles gambiae* seems to recognize blood groups and feed preferentially on group O (Panda*et al.,* 2012). The basis of this recognition is unknown, but may be related to the occurrence of ABO substances on skin cells and in sweat secretions (Panda*et al.,* 2012). This latter explanation reflects the higher number of parasitaemia seen among blood group O in this study.

The results of study in Odisha, India, indicated that blood group O was associated with reduced and B blood with increased risk of development of severe malaria (Panda*et al.,* 2012).Similarly, Ito *et al.* (2014) in Delta state-Nigeria,reported results which agreed with that found in this study, in which, blood group O was associated with 85.71% susceptibility to malaria infection and was the most susceptible of the blood groups.

Finally, there is a possible relationship between the ABO blood type and genotypes of individuals and susceptibility to certain diseases.Zhang *et al*. (2014), reported that blood group A was found to confer increased risk of gastric, pancreatic, breast, ovarian and nasopharyngeal cancers. While blood group O was found to be linked to decreased risk of gastric, pancreatic, breast, colorectal, ovarian, esophagus and nasopharyngeal cancers. In this study however, the HbAS (carrier) genotype had the highest frequency of cancers although among the normal genotype,there were higher cases of Breast and Skin cancers. However, there was no statistical association found for this observation among the selected patients that formed this study population (Appendix VI).

CHAPTER SIX

6.0 CONCLUSION

Although the study set out to determine all *plasmodium* spp. possibly present in the study area and its environs, results found in this study indicated the presence and prevalence of only a single species,*P. falciparum.* The prevalence of malaria in the study area was found to be 63.4%. The parasite density among the study population was reported as low (26.5%), moderate (15.4%) and high (21.5%).

Furthermore, the study established a 100% prevalence of EBV in the study area. It further established higher ISRs among patients who had both cancer and malaria (1.34) and lower levels among febrile patients who had no malaria (0.59). There was a seven-fold increased chance of severe malaria parasitaemia progressing into an EBV-related cancer established in this study.

The co-infection of *falciparum* malaria and EBV among patients in this study was 63.4%.The co-infection between EBV and *falciparum* malaria was such that higher malaria parasite densities corresponded to higher ISRs among patients.Malaria and EBV co-infection was higher among the sickle-cell variant, HbAC (100%) and normal genotype, HbAA(93.7%) and loweramong the sickle-cell genotype, HbSS (76.9%) and the sickle-cell career, HbAS (88.0%)indicating some level of protection enjoyed by the sickle-cell trait against the co-infection.

Finally, exposure to X-rays, even during routine medical investigations and a family history of cancer were found in this study to be the risk factors most predisposing to EBV-related cancers.

6.1 RECOMMENDATIONS

Given the high prevalence of *falciparum* malaria obtained in this study, the Nigerian government should strive to join the list of African countries cleared by the World Health Organization to have eradicated malaria within their borders. This can be achieved throughpublic health educationand awareness campaigns for mothers and health care givers.As a preventive measure,

free or subsidized Insecticide Treated bed Nets (ITNs) should be made available to households all over the country so as to control the infection of malaria in women and children, since they have been shown to be the most affected. Governments should train more health workers who can go into the rural areas to enlighten rural dwellers about malaria prevention and control. Children should also be treated with antimalarial drugs every three months to prevent malaria and to kill (if any) the early stage of malaria parasite.

Relevant agencies in the ongoing fight to roll back malaria must also intensify their efforts not only in providing effective therapy for the infected but to also provide adequate control and preventive measures for the uninfected. Further work needs to be done to establish the role of coinfection with *falciparum* malaria and EBV in carcinogenesis. Since there are no licensed vaccines against malaria(WHO, 2015),it is time for the malaria vaccine to be developed and made availableas*Plasmodium falciparum* is increasingly becoming associated with EBV-related cancers and other pathological conditions.

Many individuals are usually unaware of their clinical status blood group type and genotype,subsequent researchers should be prepared to run those tests themselves in order to enhance better research results, since the questionnaire was used to obtain such data in this study.

Finally, in this studythe seemingly innocent exposure to X-rays during routine medical investigations posed the greatest risk of carcinogenesis (more than even a family history of cancer) patients who already have an underlying co-infection with EBV and *falciparum* malaria.Better investigative tools with the least possible harm to patients should be developed in order to avoid the dangers of the current ones being used.

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Appendix I: Ethical Approval for study

HEALTH RESEARCH ETHICS COMMITTEE

AHMADU BELLO UNIVERSITY TEACHING HOSPITAL

SHIKA - ZARIA, NIGERIA.

E-mail: abuthshika@yahoo.com website: www.abuth.org

Chairman of Board: Chief. Shuaib Oyedokun Afolabi Fnil

Chief Medical Director: Prof. Lawal Khalid, MBBS, FMCS, FWACS, FRCS(ED) mnl

Chairman, Medical Advisory Committee: Prof. Abdullahi Mohammed, MBBS, FWACP, FICS

Director of Administration: Barr. Ishak Bello, LL.B, BL., LL.M, PGDM, AHAN, FCAI

Our Ref: ABUTH/HREC/CL/05 Date: 18th July, 2016

Your Ref:

ABUTH HREC FULL ETHICAL CLEARANCE CERTIFICATE

Serological Tumor Markers specific for Epstein - Barr virus in cancer and non-cancer patients attending ABUTH, Zaria Nigeria.

ABUTH Ethics Committee assigned number:	-	ABUTHZ/HREC/Q7 / 2015.
Name of the principal Investigator:	-	**Mr. Momoh Sati Joseph**
Address of the Principal Investigator:	-	Dept. of Microbiology, ABU Zaria
Date of receipt of valid application:	-	6Th July, 2015
Date of meeting when final determination		
On Ethical approval was made:	-	1st September, 2015

This is to inform you that the research described in the submitted protocol, the consent forms and other participant information materials have been reviewed and given **full approval by the Health Research Ethics Committee.**

Please note: this approval dates from **18Th July, 2016 – 18Th July, 2017**

No participant recruitment into this research may be conducted outside these dates.

All informed consent forms in this study must carry the ABUTH HREC number assigned to this research and the duration of ABUTH HREC approval of the study.

This HREC expects that you submit your application as well as an annual report for ethical clearance renewal 3 months prior to expiration of study dates. This is to enable you obtain renewal of your approval and avoid interruption of your research.

If there is delay in starting the research, please inform the ABUTH HREC so that starting dates can be adjusted accordingly.

No changes are permitted in the research without prior approval by ABUTH HREC, except in circumstances outlined in national code for Health Research Ethics: **http://www.nhrec.net.**

ABUTH HREC reserves the right to conduct compliance assessment visits to your research site without prior notification.

Prof. Aisha.I. Mamman MBBS, FMCPath
Chairman, ABUTH HREC

Appendix II: INFORMED CONSENT FORM

My name is Momoh, Sati Joseph from the Department of Microbiology, Ahmadu Bello University, Zaria. I am researching on the Prevalence of *Plasmodium* species and co-infection with Epstein-Barr virus among selected patients attending Ahmadu Bello University Teaching Hospital, Shika, Zaria-Nigeria.

Serial No: ……. Hospital No: …… Phone No: ……..
Having been well-educated by the researcher on the concept, importance as well as steps that will be taken to carry out this research;

I……..... ……. hereby willingly accept to be enrolled in this study, being made to understand that;
The data I will provide is solely for the purpose of this research work and confidentiality is assured, and that the blood sample collected will be used for serological analysis and *Plasmodium* spp. detection respectively, and the result will be communicated to me if I so desire.

…… ……
Signature/thumbprint of client with date Signature of witness with date

Appendix III: Questionnaire

Section A: Biodata

1. Sex? [Male] [Female]
2. Age? [0-10] [11-20] [21-30] [31-40] [41-50] [51-60]
3. Occupation? …….
4. Address? …….
5. Education Status? [Primary] [Secondary] [Tertiary]
6. Marital Status? [Single] [Married] [Divorced]

Section B: Clinical Data

1. Blood Group? [A] [B] [AB] [O]
2. Genotype? [AA] [AS] [SS] [Others]
3. Frequency of malaria fever? [Once monthly] [Once in six months] [Once a year] [Others].
4. Do you have cancer? If yes, what type?.....
5. Does anyone in your nuclear or extended family have cancer? [Yes] [No]

Section C: Likely Risk Factors for Cancer

1. Do you use oral contraceptives? [Yes] [No]
2. Do you smoke? [Yes] [No]
3. Do you take alcohol? [Yes] [No]
4. Have you had x-ray scan before? [Yes] [No]
5. If yes, how many times? .....
6. Do you use body-bleaching creams? [Yes] [No]

Appendix IV: ELISA Result Sheet I

	1	2	3	4	5	6
A	0.128	2.265	0.043	1.633	1.379	1.668
B	1.969	2.140	2.046	1.866	2.123	1.914
C	2.546	0.591	2.865	1.962	1.970	0.104
D	2.657	2.376	2.733	0.562	0.794	2.110
E	0.288	2.401	0.951	2.471	2.836	0.169
F	2.556	1.857	2.387	2.511	2.340	1.896
G	2.520	0.860	2.420	2.512	1.448	0.987
H	2.383	0.770	1.631	2.229	1.769	2.220

Appendix IV cont'd: ELISA Result Sheet II

	7	8	9	10	11	12
A	1.422	1.967	1.135	1.149	1.144	1.748
B	1.723	2.018	1.756	1.406	1.292	1.470
C	2.256	2.247	2.265	2.182	0.646	1.924
D	1.627	1.957	2.158	0.526	1.788	1.974
E	1.762	0.227	1.503	1.406	1.570	1.997
F	2.097	2.387	0.818	2.152	1.795	1.559
G	2.090	0.654	1.995	0.735	1.124	1.657
H	1.769	2.075	1.750	1.961	1.350	1.858

Appendix IV cont'd: ELISA Result Sheet III

	1	2	3	4	5	6
A	0.113	2.051	0.044	2.512	2.084	2.140
B	1.188	1.532	1.582	0.417	1.788	1.803
C	1.801	0.000	2.115	1.860	1.972	2.249
D	1.966	1.751	1.413	1.976	1.858	1.942
E	1.870	1.631	1.844	1.156	2.312	1.547
F	1.897	1.670	1.843	0.224	1.349	1.315
G	0.618	1.527	1.493	2.087	1.085	0.000
H	2.190	2.023	2.162	1.862	2.015	2.129

Appendix IV cont'd: ELISA Result Sheet IV

	7	8	9	10	11	12
A	1.761	1.769	2.032	0.268	1.726	1.473
B	2.109	1.068	1.803	1.818	1.780	1.544
C	2.109	2.102	1.874	1.986	1.405	1.428
D	1.814	0.146	1.846	2.059	1.979	2.073
E	1.811	2.103	1.090	1.913	1.758	1.947
F	1.974	0.184	1.848	2.156	1.930	1.306
G	2.071	2.014	1.936	1.739	1.729	2.052
H	2.073	1.156	0.024	1.221	1.872	1.091

Appendix V:

Table 4.15: Relationship between ABO blood type and Cancer among the selected patients attending ABUTH, Zaria.

Group		Number Examined	ABO Blood Type A (%)	B (%)	AB (%)	O (%)	Do not know (%)
	Breast	21	5 (23.8)	2 (9.5)	2 (9.5)	7 (33.3)	5 (23.8)
	Cervical	11	3 (27.3)	1 (9.1)	2 (18.2)	2 (18.2)	3 (27.3)
Cancer:	Head & Neck	18	3 (16.7)	6 (33.3)	1 (5.6)	4 (22.2)	4 (22.2)
	Intestinal	6	5 (83.3)	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)
	Lung	4	1 (25.0)	1 (25.0)	0 (0.0)	0 (0.0)	2 (50.0)
	Prostate	2	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (100.0)
	Skin	14	3 (21.4)	4 (28.6)	1 (7.1)	2 (14.3)	4 (28.6)
No Cancer:		118	39 (33.1)	41 (34.8)	11 (9.3)	47 (39.8)	65 (55.1)
Total		194	59 (30.4)	55 (28.4)	17 (8.8)	63 (32.5)	85 (30.5)

χ^2=63.13; df=1; P=0.009