KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY,

KUMASI

COLLEGE OF SCIENCE

DEPARTMENT OF BIOCHEMISTRY AND BIOTECHNOLOGY

DETECTING AND QUANTIFYING *PLASMODIUM FALCIPARUM* IN BLOOD AND TONSILS: TOWARDS AN UNDERSTANDING OF MALARIA-RELATED ONCOGENESIS

BY

STEPHEN KUSI

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DECLARATION

I hereby certify that this thesis, which I now submit toward the award of Master of Science in Biotechnology, is entirely the record of my own work, that the work is original and does not contain any material from other people's work, except where such work has been cited and acknowledged in the text of my work.

Stephen Kusi	……..……	……..
Student (PG)	Signature	Date
Prof. Karen A. Duca	……	……
Supervisor	Signature	Date
……..	……	……
Head of Department	Signature	Date

DEDICATION

I dedicate this work to my parents, the Reverend and Mrs. Dickson Donkor

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ABSTRACT

Epidemiological evidence strongly implicates chronic *Plasmodium falciparum* infection in the aetiology of endemic Burkitt's lymphoma, although the role of malaria is still not well understood. A characteristic feature of this tumour is a chromosomal translocation in which a pro-cancer gene, *MYC,* is juxtaposed to the promoter region of the immunoglobulin heavy chain gene, leading to the uncontrolled growth of B-lymphocytes. This study was designed to test whether the malaria parasite actually resides in tonsils and can directly cause DNA damage which could then predispose to the Burkitt's lymphoma translocation. Three methods of diagnosis, namely microscopy, rapid diagnostic test (RDT) and qPCR, were used to detect and quantify *P. falciparum* in blood and tonsils obtained from tonsillectomy patients. Interestingly, parasitemia was detected in tonsils by all three methods, even in cases where parasitemia was negative with whole blood. Comet assay was then performed for each sample to estimate DNA damage. DNA damage was assessed in terms of tail DNA, tail length and tail moment. Consistent with the hypothesis, student t-tests revealed significant differences in DNA damage between low- and high-probability parasitemia samples by all three comet parameters assessed (p-values 0.0266, 0.0316 and 0.0389 respectively; alpha =0.05). These data demonstrate that *P. falciparum* is a potent mutagen and suggest that the parasite might directly cause the characteristic translocation of endemic Burkitt's lymphoma.

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DNA	-	Deoxyribonucleic acid
eBL	-	endemic Burkitt's Lymphoma
HRP-2	-	Histidine rich protein-2
MNC	-	Mononuclear Cells
MYC	-	Myelocytomatosis cellular oncogene (also called c-MYC)
PBMCs	-	Peripheral blood mononuclear cells
PBSA	-	Phosphate buffered saline albumin
qPCR	-	quantitative Ploymerase Chain Reaction
RDT	-	Rapid diagnostic test

CHAPTER ONE

INTRODUCTION

1.1 Background

The concept that malaria might be causally associated with cancers has been posited since the turn of the 19th century. Dr. Friedrich Loffler, the discoverer of the diphtheria bacillus and a leading germ theorist at the time, postulated that this association was rather an inverse one. He believed that malaria infection prevented cancer (Robinson, 1903). Today, however, growing epidemiological evidence intricately ties malaria to cancers. First, the two diseases have been reported as showing parallel epidemiological trends (Cardy *et al*., 2001). A recent study also found strong association between malaria incidence and all cancer mortality in some states in the US (Lehrer, 2010). Suresh and colleagues observed common biophysical properties between tumour cells and *Plasmodium* infected red blood cells (Suresh *et al*., 2005) and the anti-malarial drug Artesunate reportedly possesses anticancer effects (Efferth *et al*., 2001).

The malaria disease, particularly *P. falciparum* malaria, is known to exert a myriad of immunopathologic effects (Autino *et al*., 2012; Higgins *et al*., 2011). Immunosupression is one such effect. This has been postulated as the role played by malaria in the aetiology of a jaw cancer most commonly found among children in the malaria-holoendemic sub-Saharan Africa, viz. endemic Burkitt's lymphoma (eBL), but is it so? Immunosupression, it has been shown, favours the production of the cytokines interleukin 4 (IL-4) and interleukin 10 (IL-10) resulting in a T-helper-2 dominant immune profile, which enhances B-cell proliferation (Benjamin *et al*., 1992; Lubega,

2007). This proliferation might possibly give rise to specific cytogenetic lesions responsible for cancer development. A closely related mechanism of immunosuppression is by the inhibition of the anti-tumour immunity mediated by regulatory T cells (T-regs), which are upregulated in Epstein-Bar virus related tumours (Li *et al*, 2009) but downregulated by malaria.

However, the mechanism of immunosupression seems an improbable aetiology for the cancer. Separate studies found no positive correlation (Mbidde *et al*., 1990; Lazzi *et al*., 1998; Lucas *et al*., 1994) between [childhood] HIV infection (clearly highly immunosuppressive) and Burkitt's lymphoma. Secondly, the molecular characteristic of the tumour suggests a mechanism other than, or accessory to, immunosupression.

A defining characteristic of 97% of eBL tumours is a chromosomal translocation involving a shift of the human *MYC* proto-oncogene (a regulator gene encoding a transcription factor; and a mutation in which might result in cancer) into an Ig gene locus in back of the promoter and enhancer regions. This new location for *MYC* confers a proliferative feature to the B-cell thereby rendering it more likely to accumulate further mutations. Three translocations have been observed so far, with a particular translocation, designated as t(8;14), by far the most common. This event likely takes place when naïve B-cells undergo the germinal centre (GC) reaction within tonsils, as eBL tumour cells retain a GC phenotype. Interestingly, the breakpoints on chromosome 8 where *MYC* is located are unique in eBL and are quite different than in either sporadic BL (sBL) or the HIV-associated BL (HIV-BL), the other two subtypes of BL. The break occurs generally far upstream of exon 1 in eBL and just before exon 2 in the other

forms. These breakpoints are correlated with both geography and the degree to which a ubiquitous human herpesvirus, Epstein-Barr virus (EBV) is tumour-associated (Barriga *et al*., 1988; Gutierrez *et al*., 1992; Pelicci *et al*., 1986; Shiramizu *et al*., 1991). Further upstream breakpoints of the exon1 of the *MYC* gene are EBV-associated in Africa, but infrequent in South America, where there is much less *P. falciparum* malaria. Such breakpoints do not occur at all in North American and European sporadic BL. *Plasmodium falciparum* is common to both environments where far 5' breaks occur. These observations suggest that something more direct than immunosuppression is at work.

The nuclear enzyme known as activation-induced cytidine deaminase (AID) catalyzes the introduction of double stranded breaks in DNA during class switch recombination and somatic hypermutation, processes essential for antibody diversification. AID is a potent mutagen, especially when dysregulated. Using a mouse model, Nussenzweig and others have demonstrated that overexpression of this enzyme is both necessary and sufficient to induce the mouse-equivalent of the t(8;14) translocation (Nussenweig and Nussenweig, 2010; Ramiro *et al*., 2007; Robbiani *et al*., 2008; Wang *et al*., 2009). EBV is also known to upregulate AID expression, as can HIV when it picks up the CD40 ligand on the virion surface that is exiting a T-cell (Epeldegui *et al*., 2010; Roughan and Thorley-Lawson, 2009; Epeldegui *et al*., 2007). As of now, there has been no published report showing that malaria alone upregulates AID.

The question then becomes whether malaria is capable of inducing chromosomal breaks. In order for this to happen, there must be some interaction between *Plasmodium falciparum* and the B-cell. *P. falciparum* is known to promote chromosomal breakage in hyper-reactive malarial splenomegaly (HMS) that can lead to lymphomas (Bates *et al*., 1991). The question then is: Could it be so for eBL? Chene and colleagues (2007) have shown that malaria is capable of binding on the surface of lymphocytes and cross-linking B-cell receptors, thereby reactivating EBV replication. EBV loads, however, can be much higher than those induced by this cross-linking in other conditions that do not lead to eBL. So, this is not an adequate explanation of eBL pathogenesis (Chene *et al*., 2007). It is probable that malaria in the tonsils acts more directly to facilitate the translocation setting the stage for lymphomagenesis. Several mechanisms are possible: (1) a malarial protein may enter the nucleus, associate with the AID-mutasome and direct the translocation during the normal process of AID-mediated somatic (or V(D)J) and class switch recombinations; (2) a malarial kinase may hyperactivate AID, leading to its mutagenic activity; (3) chronic infection with malaria may lead to hyperactivated AID and unremitting stimulation of B-cells latently infected with EBV.

Incidentally, there is yet an often-overlooked but potent source of DNA damage that takes place as part of normal host immune responses against the malaria parasite. During inflammatory processes in the course of malaria infection, one crucial line of anti-parasite defense is the generation of free radicals (Narsaria *et al*., 2012). These radicals exert a genotoxic effect on host cells, among other cellular injuries. Also, such drugs used in anti-malaria chemotherapy as chloroquine, primaquine and derivatives of

artemisinin are all known inducers of free radical production (Percario *et al*., 2012) and potentially give rise to genetic damage.

Thus, malaria can potentially trigger DNA damage by a variety of mechanisms. The eventual genetic instability might then seed cancer development, since DNA damage is the ***sine*** *qua non* for cancer development (Kari, 2007), as evidenced by the characteristic translocations of BL. Although intense *P. falciparum* malaria transmission, rather than hyper-parasitemia, is classically associated with the malaria-associated cancer in Burkitt's lymphoma, higher parasitemias are positively correlated with disease severity in endemic areas (Tangpukdee *et al*., 2012). Thus, it may be interesting to investigate the association between *P. falciparum* parasitemia and chromosomal breakage. In this study the hypothesis that malaria parasitemia is associated with global DNA damage was also tested.

1.2 Problem Statement

Chronic *P. falciparum* malaria can result in a wide range of pathologies, including cancers. The most severe pathologies of the disease are often associated with high intensity of disease transmission. DNA damage, which potentially leads to mutagenesis or development of genetic mutations, is one of the probable phenomenona in high malaria transmission areas such as Ghana. Cancers, on the other hand, irrespective of their origin or nature, arise from a damaged genome. Thus, it is likely that genomic instability might result from *P. falciparum* infection.

1.3 Main Objective

This study aimed to investigate whether there exist a correlation between *P. falciparum* parasitemia and global DNA damage.

1.4 Specific Objectives

To achieve the set aim, it was crucial to first show that the malaria parasite actually resides in tonsils from where cellular events potentially lead to DNA damage, setting a stage for malaria-related oncogenesis. Thus, the two specific objectives were:

1: To Detect and Quantify *P. falciparum* in Blood and Tonsils.

Microscopy, rapid diagnostic tests (based on immunochromatography) and qPCR were used to detect whole parasites, antigens, and parasite DNA respectively.

2: To detect DNA Damage in Mononuclear Cells

The 2D comet assay (single cell 2D gel electrophoresis) was used to assay for DNA damage.

1.5 Justification

The epidemiological evidence linking *P. falciparum* malaria and endemic Burkitt's lymphoma is so strong that it has been predicted that effective control of the parasite transmission in holoendemic Africa (including Ghana) would result in a significant reduction of the eBL in sub-Saharan Africa (Thorley-Lawson and Allday, 2008). Although it is still not clear what the role of malaria is precisely, it is clear that a genetic instability is a key initiating factor in tumour development. Emerging evidence suggests that upregulation of AID results in the hallmark chromosomal translocation.

The additional possible mechanism of genetic damage by oxidative stress, a common feature of the malaria infection, cannot be ruled out, although it might not directly cause translocations. It is known that intense *P. falciparum* transmission, rather than hyper-parasitemia, is associated with cancer risk in the case of the classical malaria-associated cancer, eBL. Thus, it is likely that regardless of the upstream events, *P. falciparum* malaria parasitemia, either cooperatively or solely, is responsible for the DNA damage in eBL. Therefore its DNA damaging effects might be gauged by assessing the level of DNA damage in tonsils. This study was designed to determine whether the *P. falciparum* parasite could be detected in tonsils and whether parasitemia is associated with DNA damage in mononuclear cells.

CHAPTER TWO

LITERATUREREVIEW

2.1 The Malaria Disease.

2.1.1 General Biology of the *Plasmodium* Parasite

Malaria remains a global health problem, despite increasing efforts at controlling the disease. It is caused by an apicomplexan parasite of the genus *Plasmodium*. To date, four (4) species are known to infect humans, namely *Plasmodium falciparum*, *P. vivax*, *P. ovale* and *P. malariae* (Antinori *et al*., 2012). *Plasmodium knowlesi* is an emerging fifth species (Myrvang, 2010) capable of causing malaria in humans, but more commonly in monkeys. Of the four species, *P. falciparum* is the leading cause of malarial illness in sub-Saharan Africa and causes the most severe form of malaria, celebral malaria, which is characterised by neurological dysfunction and coma. It is estimated that over a million deaths are caused by malaria (mainly by *P. falciparum* malaria) across the globe annually (Murray *et al*, 2012). Poor, tropical countries remain the most afflicted due to socio-climatic factors such as lack of clean environment, hot and humid climate that favour the breeding of the vector insect, the female anopheles mosquito. Consequently, there is a perennial and intense transmission (holoendemicity) of the parasite among all age groups of the population in the sub-tropics, including Ghana (de-Graft Aikins, 2007).

The *Plasmodium* parasite is a eukaryotic intracellular parasite belonging to the phylum Apicomplexa. Other notable members are *Cryptosporidium* and *Toxoplasma* (in humans), *Babesia* and *Theileria* (in cattle) and *Eimeria* (in poultry). This group of

organisms are characterized by a set of organelles situated at the apex (hence the name "apical") of the parasite at certain stages of its life cycle. In the case of *Plasmodium* species there are three invasive stages, viz. sporozoite, merozoite and ookinete stages. These apical organelles include specialized secretory organells, namely rhoptries, micronemes and dense granules which are employed in parasite invasion of host cells.

The *Plasmodium* parasite has a complex life cycle involving two separate phases of asexual and sexual reproduction occurring alternately in the human host and the mosquito vector. During the asexual phase, the parasites at a developmental stage known as sporozoites are injected into the bloodstream of the human host during a bite of the female anopheles mosquito carrying the parasite. Once inside the body, the infective sporozoites are carried by the blood stream to the liver where they infect hepatocytes in the liver, replicate as schizonts in a period lasting for about 10-14 days. The now mature schizonts burst to release thousands of merozoites ready to invade red blood cells or erythrocytes to initiate the cycle known as the erythrocytic stage of the parasite (Figure 1). It is important to emphasize that Apicomplexans have a notable capacity for invading host cells using the apical apparatus described above. As an instance, invasion takes place through four steps in the case of *Plasmodium* (Gratzer and Dluzewski 1993; Mitchell, 2004), and typical invasive mechanism is demonstrated in the invasion of erythrocytes by merozoites.

Upon coming into contact with a red blood cell (or erythrocyte), interaction between merozoite surface proteins (such as the merozoite surface protein-1, MSP-1) and the

erythrocytes leads to primary and secondary proteolytic processing activities that result in merozoite attachment to the erythrocyte. This is followed by the reorientation of the parasite mediated by the apical membrane antigen-1 (AMA-1) such that the apical end is positioned toward the erythrocyte membrane, transiently deforming the erythrocyte in the process. Next, the contents of the specialized secretory organelles (rhoptries, micronemes and dense granules) are then released one after the other, resulting in the formation of a junction between the parasite and host cell using proteins discharged from these secretory organelles. Finally, the parasite enters the host cell through this junction by the cellular movement known as gliding motility (Cowman and Baum, 2012).

After infecting the erythrocytes, the merozoites assume ring-shaped forms known as trophozoites and begin to feed on the red blood cells. Following a growth period, the trophozoites undergo asexual multiplication in red blood cells which become so engorged with these merozoites that they burst to free the new merozoites which will then reinvade new erythrocytes in a cyclical manner, lasting 48 hours.

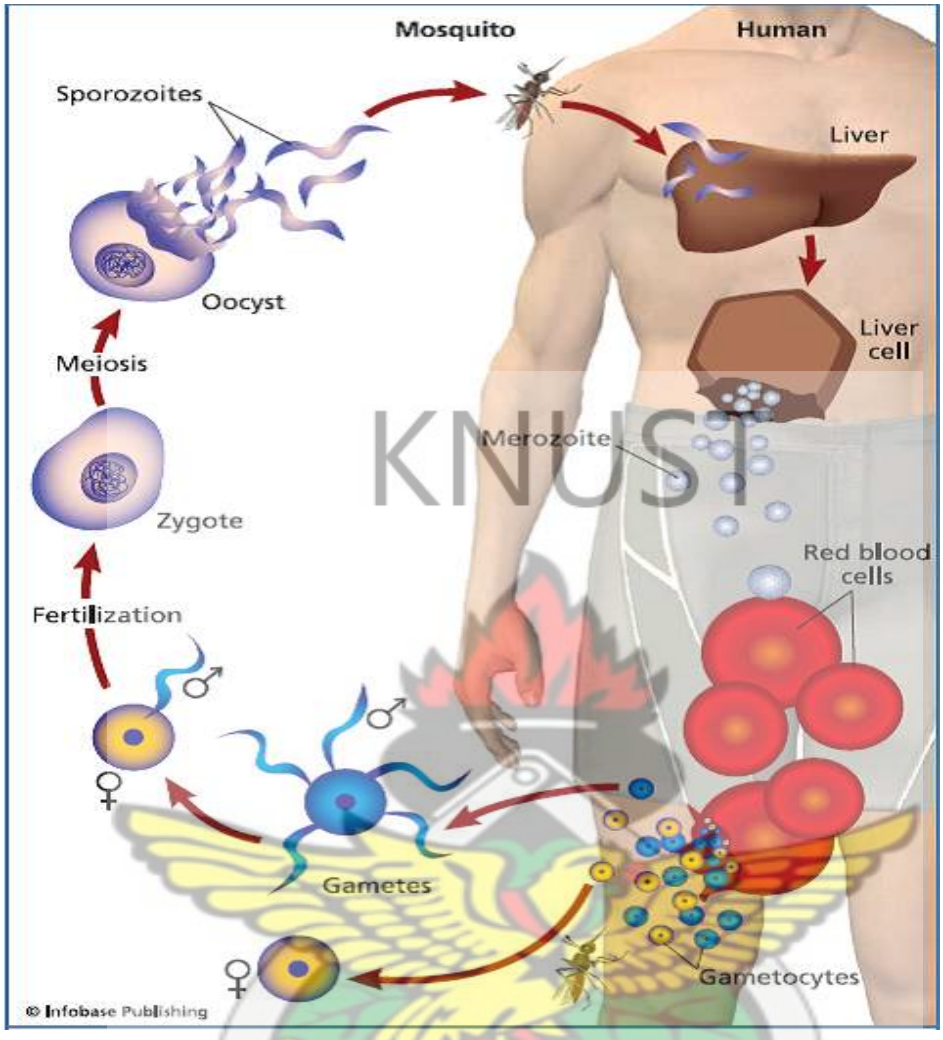

Figure 1: A simplified diagram showing the life cycle of *Plasmodium*. Infected female anopheles mosquito bites and injects saliva that contains *Plasmodium* sporozoites into the human host. After a few days, the sporozoites invade liver cells where they multiply asexually. Infected liver cells burst to release merozoites that infect red blood cells. Merozoites then reproduce asexually inside red blood cells which eventually burst to release new merozoites that re-infect new red blood cells, whiles others release gametes that can infect mosquitoes. Female anopheles mosquito bites a human infected with malaria and picks up *Plasmodium* gametocytes which fertilize and undergo developmental process culminating in sporozoites residing in the salivary glands of the mosquito. Source: Marcus B A (Ed.) (2009). Deadly Diseases and Epidemics: Malaria (2nd edition). Reprinted with the permission of the Publisher (see Appendix).

A sexually differentiated generation of the merozoites emerges after several rounds of mitotic divisions in the red blood cells that give rise to male and female gametocytes. A mosquito taking a blood meal at this stage of the parasite's life cycle from a human host picks up these gametocytes which soon mature into gametes in the mid-gut eventually resulting in fertilization. Zygotes formed further develop into motile ookinetes, penetrate the gut epithelium to become circular oocysts, and finally into elongated sporozoites which find their way into the salivary glands of the mosquito, from where they enter new host during a mosquito bite.

The clinical manifestations of malaria range from mild, uncomplicated malaria characterized by headache, vomiting, abdominal pains, nausea, fever and so on, to severe and fatal disease conditions such as celebral malaria, anaemia and respiratory distress. Severe *P. falciparum* malaria is distinguished from uncomplicated malaria by the evidence of vital organ dysfunction (WHO, 2010). Celebral malaria is marked by coma and results when *Plasmodium falciparum* crosses the blood-brain barrier. Consequent interruptions in perfusion lead to a hypoxic condition, which in turn gives rise to nerve failure. Respiratory distress results from a similar mechanism of pathogenesis. Rossetting (spontaneous binding of uninfected erythrocytes to infected erythrocytes) and cytoadherence (adherence of *P. falciparum*-infected erythrocytes to the endothelium) of severe *P. falciparum* infection lead to the destruction of endothelial cells and subsequently to a reduction in perfusion and then oxygen debt which results in shortness of breath (English *et al*., 1997). In pregnant women the development of the placenta provides receptors for infected red cells expressing parasite antigens to latch

onto and become sequestered in the microvasculature. There the parasites flourish in the hypoxic pockets and escape splenic clearance (Costa *et al*., 2006; Rogerson *et al*., 2007). This could have severe consequences for both mother and foetus, including low birth weight, maternal anaemia and even death (Desai *et al*., 2007).

The malaria disease can be treated upon diagnosis by chemotherapy. In order to prevent parasite resistance to drugs, whiles improving treatment outcomes, a combined drug therapy is recommended by WHO for treating malaria. Artemisinin-combination therapy (ACT) is currently the most effective treatment strategy for uncomplicated *P. falciparum* malaria (WHO, 2010). This involves the use of an artemisinin derivative such as artesunate in combination with other anti-malaria drugs such as amodiaquine. Prevention of the disease may be achieved by chemoprophylaxis and by keeping oneself from exposure to the mosquito vector.

A key stage in the pathogenesis of malaria is the acute lysis of red blood cells, symptomized by anaemia (Fendel *et al*., 2010), fever and chills (Bouldouyre *et al*., 2006). Hemozoin is a bi-product of this breakdown which takes place inside the food vacuole of the malaria parasite. This pigment is gaining increasing interest in recent times owing to its possible mediator role in pathogenesis. It binds to toll-like receptor 9 (TLR-9) to a pro-inflammatory cascade (Pichyangkul *et al*., 2004). Disease severity in malaria is correlated with the accumulation of heme in organs such as the liver, spleen and brain (Nguyen *et al*., 1995).

In the course of an infection, the *Plasmodium* parasite employs complex life stage conversions that all culminate in the breaking of host immune memory. Members of the *var* gene encoded Erythrocyte Membrane Protein 1 (PfEMP1) family are expressed on the surface of infected red cells and play a role as virulence factors mediating cytoadherence. They are also targets of host antibodies. There are about 60 genes in this family, with only one gene transcribed at any given time. As an evasive strategy, the parasite may randomly switch to different antigens at different stages in a process known as antigenic variation (Rasti *et al*., 2004).

2.1.2 Immunity to Malaria Infection

Immunity to malaria depends on factors such as age, history of infection with malaria, host genetics and the malaria transmission intensity of location. Young children less than six months depend passively on maternal anti-malarial antibodies. Therefore the risk of severe malaria or death is highest during this stage of development. There are a number of innate mechanisms that prevent malaria infection, independent of whether or not there has been a previous infection. Evidence available suggests that in high transmission areas, for example, selection pressure drives the emergence of certain genetic polymorphisms that confer protection against severe *P. falciparum* malaria. Examples of these polymorphisms include hemoglobinopathies such as the sickle cell trait and (Doumbo *et al*, 1992; Kreuels *et al*, 2010) and certain thalassemias (Veenemans *et al*, 2008; Than *et al*, 2005). Some of the host genetic factors conferring natural immunity to malaria are the defective polymorphisms of the fc-gamma R2b (fcgR2b) receptor gene of phagocytic cells such as macrophages (Willcocks *et al*, 2010),

and the glucose-6-phosphate dehydrogenase (G6PD) deficiency gene (Luzzatto, 2012). Duffy negativity which is reported to be widespread in Africa also confers protection against *P. vivax* malaria (de Carvalho and de Carvalho, 2011).

Certain lymphocyte subsets of the innate immune system, such as extrathymic T cells [Natural Killer (NK) 1.1^{-} and intermediate TCR (TCR^{inter}) cells] and auto-antibody-producing B1 cells, are associated with protection against severe malaria (Schmieg *et al*, 2003. These cells are derived from the liver, the intestine and other immune organs, unlike conventional T and B cells which mature in the thymus and bone marrow respectively. It has been shown that these cells are activated during the acute phase of malaria infection to curtail disease progression. Natural Killer cells have the capacity to lyse *P. falciparum* infected erythrocytes and can also induce the production of interferon-gamma (IFNγ) which activates macrophages to engulf the parasitized erythrocytes. On their own, other phagocytic cells are able to instigate an immune response against infected erythrocytes.

The process of acquiring active immunity is initiated following a primary exposure to the malaria parasite. People living in malaria high transmission (holoendemic) areas develop specific immunity progressively but gradually, through repeated infections (Hafalla *et al*., 2011). By age five, children in these areas would have developed immunity to severe malaria but not uncomplicated malaria; and by adulthood, most people can clear parasites without treatment (Bruce *et al*, 2000; McGregor, 1974).

The refractoriness of malaria is mainly due to the existence of genetic and antigenic variants of the parasite which imposes a huge challenge on the immune system to produce antibodies specific for all parasite antigens. Chronic infection with malaria exerts subversive effects on host immune response and causes a break in immune memory. The parasite hinders the differentiation of long-life memory B-cells, but favours short-life B-cell development (Weiss *et al*, 2010).

Malaria infection is associated with elevated levels of IgG, IgM and IgE immunoglobulin isotypes. The former immunoglobulins are associated with anti-malarial protection either by inhibiting merozoite invasion of erythrocytes and growth of asexual blood stage parasites, or by enhancing splenic clearance of parasitized erythrocytes and their sequestration in microvessels, or by opsonizing for phagocytosis and cytotoxic inhibition of infected erythrocytes by effector cells (e.g. neutrophils and macrophages). The IgE, on the other hand, is associated with a shift from a T heper-1 to a T helper-2 response, mediated by Tregs plausibly due to overproduction of Tumour Necrosis Factor (TNF) and nitric oxide (NO) caused by IgE-containing immune complexes (Perlmann and Troye-Blomberg, 2002).

CD4 T-cells play a critical role in intraerythrocytic stage cell-mediated immunity. CD8 T-cells play no such role at this stage due to the lack of major histocompatibility complex (MHC) molecules on red blood cells. However, CD8 T cells are known to play crucial roles in pre-erythrocytic immunity and are also thought to regulate immunosupression and down-modulation of inflammatory responses. Homozoin-

containing infected erythrocytes prevent maturation of dendritic cells (regarded as "professional" antigen presenting cells) thereby reducing their co-stimulation with T cells (Hisaedaa *et al*, 2005). As a result, the dendritic cells release interleukin-10 instead of interleukin-12, again driving a shift to T helper 2 (anti-inflammatory) responses. This situation gives rise to immunosupression which in turn raises the risk for secondary infections including EBV reactivation.

A common feature of malaria infection is hyper-gammaglobulinemia (Abele *et al*, 1965) resulting from the polyclonal activation of B-cells. It has been reported that the interaction of the cysteine-rich interdomain region 1(CIDR1) of PfEMP1 with peripheral B cells induces proliferation, an increase in B-cell size, expression of activation molecules, and secretion of immunoglobulins (IgM) and cytokines (tumour necrosis factor alpha (TNFα) and interleukin 6 (IL-6)) (Donati *et al*, 2004). Subsequently, a direct link between CIDR1 and EBV-infected memory B-cells was demonstrated. This observation suggests that chronic *P. falciparum* stimulation could reactivate EBV in latently infected B-cells even in the absence of acute malaria infections (Chene *et al*, 2007). This phenomenon is thought to partly explain the malaria-EBV aetiology in endemic Burkitt's lymphoma.

2.1.3 Malaria and Endemic Burkitt's Lymphoma: Emerging Research Trends

The exact nature of the interaction between *P. falciparum* malaria and EBV in the pathogenesis of the malaria-associated cancer, Burkitt's lymphoma, is poorly understood. What is certain however is a proliferative B-cell phenotype that arises out of

the dysregulation of the *MYC* proto-oncogene. Two competing, yet potentially harmonious, theories have been advanced to explain the malaria-EBV co-operativity leading to the characteristic translocation in eBL (Rochford *et al*., 2005). First, it is suggested that malaria-triggered impairment of EBV-specific T-cell responses leads to a failure by the immune system to clear EBV infected B-cells, and therefore the increased likelihood of yielding a cancerous progenitor B-cell. The other suggests that *Plasmodium falciparum* malaria induces polyclonal B-cell expansion and accordingly lytic EBV reactivation, leading to the expansion of latently infected B-cells and therefore the increased likelihood of *MYC* translocation. It has been proposed that repeated malaria infection provides the context for eBL development, regardless of the specific aetiologic route (Moormann *et al*., 2011).

A clear direction discernible from the literature is an interest in the investigation of the role of chronic *P. falciparum* infection in the development of eBL. Accordingly, recent studies suggest that chronic malaria could directly potentiate chromosomal damage (Greisman *et al*., 2012) via the toll-like receptor 9 (TLR-9) pathway (Zauner *et al*., 2010), through the upregulation of AID (Seok-Rae, 2012). There are potentially several malarial ligands, including hemozoin, which can activate TLR-9 signalling. Specifically, it has been found that by this pathway hemozoin downregulates the lytic gene expression of EBV *in vitro* (Zauner *et al*, 2010). Latent EBV infection, the stage associated with increased risk for eBL, may be promoted by TLR9 triggering via suppression of lytic EBV replication. Thus, hemozoin could act as an eBL-promoting ligand.

2.1.4 Malarial as a Potent Independent Inducer of Chromosomal Breakage

Aside the hypothesized, co-operative induction of chromosomal damage, the nature of the malaria disease itself is suspect. Oxidative stress is a common feature of *P. falicarum* infection, arising from the disturbance of the host redox state. Antioxidant defense mechanisms are crucial for quenching the free radicals especially reactive oxygen species or ROS e.g. hydroxyl radical, superoxide anion, hydrogen peroxide, and singlet oxygen; and reactive nitrogen species or RNS e.g nitric oxide, peroxinitrite and nitrogen dioxide) generated by phagocytes (macrophages and neutrophils) during inflammatory responses following a malaria infection. A delicate balance is maintained between free radical generation and their neutralization by antioxidant defense, in order to avoid cellular injury. When the free-radical side of the balance is overwhelmed by free radical overproduction, or when there is a decrease in antioxidant capacity, a state of disequilibrium arises. It is still not clear what the exact role of oxidative stress is during immune responses to malaria infection, but both harmful and beneficial effects have been claimed (Percario *et al*., 2012). It is known that the host immune system uses this as a mechanism to control the proliferation of the parasite. However, if the antioxidant versus free-radical imbalance is not repaired or is not properly restored, it invariably leads to DNA damage in the host and could eventually activate oncogenes (Eze *et al*., 1990), an end which raises a risk for oncogenesis.

Oncogenesis proceeds via three stages: 1) initiation, 2) promotion and 3) progression, and oxidative DNA damage is involved in all three stages (Aivaliotis *et al*., 2012). Initiation of DNA damage by free radicals could be by epigenetic regulation through

promoter methylation and microRNA expression (Bhattacharjee *et al*., 2013) or by the formation of 8-hydroxy-2' -deoxyguanosine (8-OHdG) (Valko *et al*., 2006). These events may lead to the promotion stage involving a number of cytogetic outcomes: base pair mutations, rearrangements, deletions, insertions and sequence amplification. The final stage of progression involves cellular responses including altering of cytoplasmic and nuclear signal transduction. Or modulation of the activity of proteins and genes that respond to stress and which act to regulate the genes that are related to cell proliferation, differentiation and apoptosis (Reuter *et al*., 2010).

Besides the endogenous sources of DNA damaging agents (oxidative stress), there are also exogenous (radiation and chemical adduction) agents of genetic damage. DNA adducts cause genetic lesions through the alkylation or cross-linking with DNA molecules (Beland and Poirier, 1994; Drablos *et al*., 2004). At present, no malarial DNA adducts have been identified. However, the recent observation that the malaria pigment hemozoin is capable of binding Plasmodial DNA (Parroche *et al*., 2007), may be suggestivetive of the adductive abilities of hemozoin. On the other hand, the capacity of malaria to generate free radicals in toxic doses has been demonstrated. Infected erythrocytes have been shown to generate free radical concentrations approximately two-fold higher than uninfected erythrocytes (Atamna and Ginsburg, 1993).

2.2 Malaria Diagnosis

The microscopic examination of stained blood films for the presence of the parasite is regarded as the method of choice for the laboratory diagnosis of malaria. However, due

to the numerous technical challenges that undermine this method, particularly in resource-poor areas (Endeshaw *et al*., 2008), alternative methods havebeen devised for routine diagnosis. Some of these limitations include low sensitivity and specificity (Snounou *et al*, 1993) and high false-negativity and false-positivity rates, under certain field conditions (Salako, *et al*, 1999). Other less commonly used methods include fluorescence techniques such as Quantitative Buffy Coat (QBC) and the Kawamoto acridine orange (KAO), both of which essentially rely on the use of a fluorochrome, usually Acridine Orange, to stain parasite nucleic acid. The QBC assay involves direct observation of centrifuged blood in capillary tube stained by acridine orange. The parasites, if present, are observed in buffy coat and red blood cell interface with cytoplasm of parasite appearing red and nuclear chromatin appearing green (Shujatullah *et al*, 2006). The KAO method is similar to standard thick smears with the exception of acridine orange staining (Kawamoto, 1991) instead of a conventional stain such as the Giemsa stain (Figure 2). These methods have been reported to exhibit the limitation of non-specificity for the different malaria species that can infect humans (Moody and Chiodini, 2000).

2.2.1 Light Microscopy

Light microscopy basically involves the staining of smears of blood on a slide with special a stain to colour the parasite's nuclei in recognizable shapes and hues, and the slide is then examined under a microscope. There are two variants of malaria smears, thick and thin smears. Thick smears involve the use of relatively larger volume of blood spread on a microscope slide into a multi-layer of dehemoglobinized (lysed) red blood

cells. The use of large volume of blood and the lysis of the blood cells improves the diagnostic outcomes. The thin smear is prepared with relatively smaller volume of blood spread in such a way as to achieve a monolayer of intact (fixed) red blood cells on the slide. This allows for identification of species. Both smears can be used to determine parasitemia. With thick smears, parasitemia is normally determined relative to white blood cells, whereas parasitemia may be determined relative to either red or white blood cells with thin smears (O'Meara *et al*, 2005).

Light microscopy is still regarded as the gold standard for diagnosis of malaria. However, under certain circumstances, especially with very low parasitemic cases and during pre-erythrocytic stages of the parasite, an uncommon level of diagnostic expertise is required in order to arrive at a valid diagnosis (Moody and Chiodini, 2000). Another notable factor that undermines the effectiveness of thin blood films or microscopic diagnosis of malaria concerns the ability of the parasite to hide in organs and tissues rather than be suspended in circulatory blood, thereby eluding detection by thin blood films (Kattenberg *et al*., 2012). Moreover, the interfering and parasite-mimicking effects of cellular/reagent debris and microscopic artifacts also pose a major hindrance to the accuracy of light microscopy (Houwen, 2002). Therefore, it is recommended that microscopy be augmented with other methods of diagnosis in order to obtain reliable results (Wongsrichanalai *et al*., 2007).

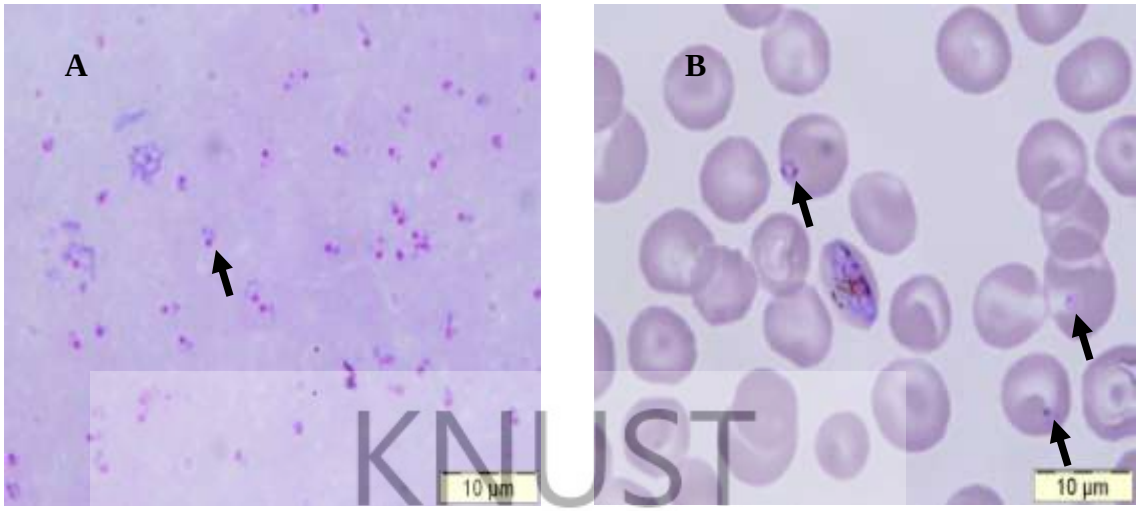

Figure 2: *P. falciparum* parasites in blood smears stained with Giemsa stain. (A). Thick smear showing numerous ring-stage parasites. (B) Thin smear showing intact red blood cells with three of them (indicated by an arrow) showing ring-stage parasite and one *P. falciparum* gametocyte (centre).

Source: Wilcox A, (1960). Manual for the microscopical diagnosis of malaria in man. Revision edition.

2.2.2 Rapid Diagnostic Testing

Rapid diagnostic tests (RDT) are immunochromatographic devices designed to target specific parasite antigen(s). They are commercially available in the form of dipstick or cassette. The two most common antigens used in RDTs are the *Plasmodium-falciparum*-specific histidine-rich protein-2 (HRP-2) and the parasite lactase dehydrogenase (pLDH), which may be either species-specific or specific to all four species of *Plasmodium* at the same time (pan-pLDH). Aldolase is another pan-specific protein target used in some RDT devices.

The general principle of the malaria RDT device is that a test sample is migrated along with a buffer solution by horizontal capillary action across the surface of a nitrocellulose membrane. Parasite antigen from blood is captured using a monoclonal antibody prepared against a malaria antigen target and conjugated to either a liposome containing

selenium dye or gold particles in a mobile phase. A second or third (in the case of dual-species diagnostic devices) capture monoclonal antibody applied to a strip of nitrocellulose acts as the immobile phase. The migration of the antigen-antibody complex in the mobile phase along the strip allows the capturing of the labelled antigen by the monoclonal antibody of the immobile phase, thus producing a visible coloured line. Incorporation of a labelled goat anti-mouse antibody capture serves as a control for the migration (Moody, 2002).

HRP-2, the most common antigen used in RDTs, is a soluble antigen of about 3kD (Wellems and Howard, 1986) secreted into plasma predominantly during the schizont rapture (Desakorn *et al*., 2005). It has the unique feature of persistence in host circulatory blood, post parasite clearance (Laurent *et al*., 2010), a feature that predisposes HRP-2 –based RDTs to false positivity. A number of findings suggest that this protein is antigenic. A recent study has shown that HRP-2 is positively correlated with disease severity (Hendriksen *et al*., 2013). It has been advanced that the pathogenic role likely played by the HRP-2 protein is the polymerization of heme to hemozoin (Choi *et al*., 1999; Sullivan *et al*., 1996).

Figure 3. Malaria RDT cassettes. Showing a negative result (left) and a positive result. A single band at the control (indicated as "C") region alone indicates negativity and the concurrence of two bands at both the control and the test (indicated as "T") regions indicates positivity. A result is deemed as invalid when a single band occurs at only the test region.
Source: Orignal, produced from this work.

RDT use is increasingly gaining popularity as an alternative to microscopy and has been advocated by WHO (2010) especially for low-resource areas. It owes its growing acceptation to its being cheap, and in some cases exhibiting higher sensitivity than microscopy (Andrade *et al*., 2010), especially in the diagnosis of pregnancy malaria (Singer *et al*., 2004). Additionally, it requires no specialized apparatus or skills, much unlike microscopy or PCR. Because antigens are carried in the circulatory blood, RDTs are able to detect an infection, in contrast to the parasite-targeting assays (microscopy and PCR), even where parasites are sequestered in organs and vascular structures such as in placental endothelia, making them more sensitive than microscopy (Mockenhaupt

et al., 2006). More importantly, however, RDTs tend to exhibit discordance with microscopy at very low parasitemic situations (Aguilar *et al*., 2012), in which cases the parasitemia is below the detection limit of the particular brand of RDT.

2.2.3 Quantitative PCR (qPCR)

The main advantages of PCR over microscopy and RDT in malaria diagnosis are sensitivity and specificity (Andrews *et al*., 2005; Snounou *et al*., 1993). Specific parasite genes are targeted and amplified to amounts sufficient to yield a band on electrophoresis. The PCR however may on very rare occasions exhibit the limitation of microscopy in being ineffectual where parasites are sequestered out of circulatory blood and requiring expensive equipment and special expertise to run (Tangpukdee *et al*., 2009). Nonetheless, the overall benefits of PCR diagnosis clearly outweigh the disadvantages.

First, misdiagnosis, which is more common with microscopy and RDTs, can lead to one of two possible outcomes: severe disease condition and possibly death, or mis-prescription of an anti-malarial drug, which can lead to the often fatal unintended drug responses on the part of the patient and drug resistance on the part of the parasite, which are all mitigated with improved diagnostic sensitivity (Wongsrichanalai *et al*., 2007). However, it is important to note that over-diagnosis is not unlikely with even qPCR. Even with malaria-immune adults, sterile immunity is never fully attained, and many healthy adults in holoendemic areas may continue to carry low-grade parasitemia in their circulatory system (Denise*et al*, 2009). Because of its high sensitivity, there is

likelihood for qPCR to falsely diagnose malaria illness for individuals who may be suffering from malaria-unrelated conditions.

2.3 Genomic Instability and Cancer Development

The unified genetic theory of cancer development or oncogenesis states that "cancer starts and ends with the malignant cell, in which genetic changes lead to constitutive activation of some genes (oncogenes) and/or inactivation of others (tumour suppressor genes), allowing that cell to evade – in all or in some microenvironments – the mechanisms controlling cell proliferation" (Ruggiero and Bustuoabad, 2006). Stated differently, the underlying instigating factor for all cancer types is genomic instability (Caburet, 2002; Eyfjord *et al*, 2005), which encompasses all genetic alterations ranging from point mutation to chromosomal translocations (Raptis and Bapat, 2006) leading to a mutation in genes regulating cell cycle checkpoints, growth arrest, DNA repair and programmed cell death (apoptosis) (Funk and Kind, 1997) and microRNA (Deng *et al*., 2008). Normally, repair enzymes constitutively attempt a repair of the alterations, but the process is itself error-prone and therefore may fail to effectively correct a genetic anomaly, resulting in a malignant phenotype.

Broadly, three main types of genomic instability have been identified so far. These are chromosomal instability (CIN), nucleotide instability (NIN), and microsatellite instability (MIN) (Lengauer *et al*., 1998). These are respectively the results of defects in DNA repair and mitotic control pathways, base and nucleotide excision repairs, and mismatch excision repairs. Xeroderma pigmentosum is caused as a result of NIN; whereas colorectal, endometrial, ovarian and gastric cancers include some of the cancers

caused as a result of MIN. Both NIN and MIN take place at the nucleotide level, whereas CIN takes place at the chromosomal level. However, either of the former can theoretically translate into the latter when the resultant defects affect genes encoding DNA repair and mitotic control enzymes (Lengauer *et al*., 1998).

CIN is particularly apposite in the present study because, along with a related condition of abnormal chromosome number (aneuploidy), it is the most common phenotype in solid tumours (Yuen and Desai, 2008; Thompson *et al*., 2010) such as in endemic Burkitt's lymphoma. In eBL, a CIN is exhibited by a hallmark translocation that involves the *MYC* proto-oncogene and the immunoglobulin heavy chain locus (IGH) resulting in the up-regulation of *MYC* expression, which leads to the lympho-proliferative B-cell phenotype (Mills *et al*, 2003), although other genetic lesions may be present in eBL. Recently, it has been discovered that a mutation (CIN) in the CD79B protein that is involved in the B-cell receptor signaling pathway was responsible for a fifth of activated B-cell-like (ABC) tumours, a subtype of non-Hodgkin's lymphomas (Davis *et al*., 2010).

2.4 The Comet Assay and Estimation of DNA Damage

The comet assay or single-cell electrophoresis has become a standard technique used for detecting and measuring DNA damage (Faust *et al*., 2004). The range of applications of this technique, first described by Östling and Johanson (1984), has been expanded over the ensuing years to provide information on the DNA-damage and repair (McKenna *et al*., 2008) in different areas in biological research including nutrition and ecology

(Nassoni, 2008; Singh *et al*., 1988). Commercially available in kits, the comet assay is simple, sensitive and cost-effective. Despite these worthy features, a few drawbacks of the assay have been noted, including the facts that there are currently no appropriate controls available for the *in vivo* comet assay (Collins, 2004) and that the comet assay is not able to detect small DNA fragments (smaller than 50kb), since these are mostly washed out during lysis and electrophoresis stages (Olive, 1999).

The working principle of the assay is similar to that of a conventional gel electrophoresis. Relatively low electric current is passed through individual cells embedded in gel matrix that is pre-coated on a glass slide. The main stages of the comet assay procedure are (1) lysis of nuclear membrane to allow movement of DNA, (2) unwinding of DNA to relax DNA supercoils, and (3) electrophoresis. During electrophoresis, the nucleoid (DNA) migrates along with the electric current in proportion to the degree of fragmentation. Intact DNA hardly moves due to its molecular heftiness; whereas damaged DNA flakes off the massive piece of DNA composing the nuclear material, forming a comet tail oriented towards the anodic region of the electrophoretic equipment. The slide is examined using a fluorescent microscope after staining the specimen with a dye post-electrophoresis; and the comets are scored based on a number of defined parameters. Numerous experimental variables affect the batch-to-batch differences in comet parameters, making the assay somewhat liable to subtle differences in these variables. These factors include concentration of the agarose gel, pH, temperature and duration of the unwinding and electrophoretic stages of the

assay protocol (Hartmann *et al*., 2003; Collins, 2004). Other factors include the voltage and amperage during electrophoresis.

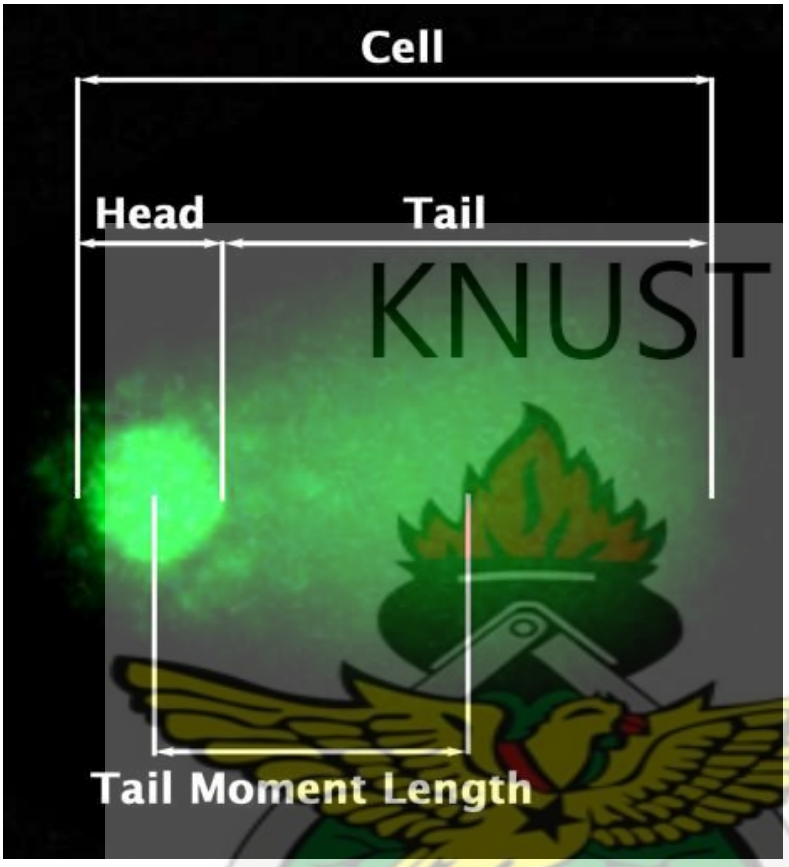

Figure 4: A comet image showing the parameters commonly used to assess DNA damage. Source: (http://portal.faf.cuni.cz)

Based on pH, two kinds of the assay exist, alkaline and neutral. Each one has a unique detection capacity. The alkaline assay, which is the preferred assay (Tice *et al*., 2000 has a pH >13. This assay detects alkaline labile sites of DNA, transient repair sites and single-stranded breaks in addition to double stranded breaks (Moller, 2006).The neutral assay (requiring solutions at neutral pH) detects only double stranded breaks, making it less sensitive than the alkaline assay (Nassoni, 2008). The overall sensitivity of the

assay is enhanced if cells are incubated with bacterial repair endonucleases that recognize specific kinds of damage in the DNA and convert lesions to DNA breaks, increasing the amount of DNA in the comet tail (Collins, 2004). In these kinds of comet assay, the comet gels are incubated with the enzyme after the lysis stage, in parallel with control gels containing enzyme buffer alone. The net enzyme-sensitivity sites are calculated by subtracting the comet score for the enzyme buffer control from the comet score for the enzyme-treated gels (Collins 2011)

The parameters that are often determined by comet scoring software are head length, tail length, [%] head DNA, [%] tail DNA, length of comet, and tail moment (the product of comet %tail DNA and tail length). The most commonly used of these to describe and assess DNA damage, however, is the percent tail DNA (Collins, 2004). This parameter, unlike the others, can be 'standardized' over different studies whiles the other metrics may not be comparable across studies (Collins *et al*., 2008). Also, this parameter covers the widest range of damage, with a linear relationship to break (damage) frequency. In the case where very low levels of damage are present, tail length is most informative. Percent head DNA is complementary to % tail DNA, and shows a decrease with increasing damage, a feature subtracting from its usefulness as data. The tail moment is sometimes favoured for its representation of tail length and tail intensity in a single value. But this apparent advantage has not always been sustainable, as it may make the dose–response curve deviate from linearity at low doses (in *in vitro* studies) (Collins, 2008). Moreover, tail moment can be calculated in different ways, and it does not have standard units.

DNA damage at the chromosomal level can be detected by standard comet assay (Collins *et al*, 2008). In this case, strand breaks and alkali-labile sites including apurinic and apyrimidinic sites, or AP sites, are the target lesions. Since this kind of assay allows for the detection of only single or double strand breaks, it may be suitable for only chromosomal instabilities (CIN). For lesion-specific measurements, such as in minisatellite (MIN) and nucleotide instabilities (NIN), a modification of the comet assay involving the use of nucleotide-specific endonuclease is required. This involves the incorporation of an additional step of digesting the DNA with a lesion-specific endonuclease following lysis of the agarose-embedded cells. The endonuclease then converts damaged bases to strand breaks (Dušinská and Collins, 1996).

In spite of the versatility of the comet assay, a determination of the exact nature of DNA damage requires additional cytogenetic methods such as the fluorescent in situ hybridization (FISH) technique (Shaposhnikov *et al*, 2011). In a typical instance, Savina and colleagues (2011) used the comet assay and FISH to respectively detect and characterize the genetic lesion responsible for the Williams-Beuren syndrome (WBS), a disorder arising from a hemizygous microdeletion at the 7q11.23 chromosomal locus of peripheral blood lymphocytes.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area, Patients and Sample Collection

Twenty-two (22) paired peripheral blood and palatine tonsil samples were obtained with the informed consent of patients undergoing tonsillectomy at the Komfo Anokye Teaching Hospital, Kumasi and the PAKS Hospital, Afrancho, near Kumasi. All participants were residents of the Kumasi Metropolis of the Ashanti Region of Ghana, although exclusion or inclusion criteria (based on age, gender, location, etc) were applied besides that a patient has to be a tonsillectomy patient. However, samples that were diagnosed by rapid diagnosis as HIV positive post-surgery were excluded as a safety measure. About 2 ml of blood samples were collected in heparinized vacutainers, whereas tonsils were collected in 50 ml Falcon® tubes containing 20 ml of PBSA (1X PBS + 0.5% w/v BSA). The samples were obtained from January-June, 2011. Blood was also spotted onto filter paper for later analysis.

3.2 Separation of Tonsillar Components

Tonsils were weighed and washed thoroughly in phosphate buffered serum albumin (PBSA) to remove residual peripheral blood. Tonsils were then minced using a surgical blade and cells subsequently dispersed into solution by gently crushing minced tonsillar bits using the plunger top of a syringe piston. Next, the cell suspension was filtered through a 40 µm pore-size BD Falcon strainer (model number 352340; BD Biosciences, USA) to remove connective tissue. Filtrate was collected in 50 ml BD Falcon tube. An aliquot of this suspension (referred to in this paper as "tonsil extract") was at this point

taken for storage to be used later for DNA isolation in qPCR assay. The remaining stock of cell suspension was then centrifuged for 5 minutes at 1200 rpm at room temperature. The supernatant was discarded and the pellet re-suspended in fresh PBSA. Twenty-five millilitres of cell suspension was carefully layered on 20 ml Ficoll solution in 50 ml tube. The tube was then centrifuged at 1200 rpm for 30 minutes without brake at room temperature. After centrifugation, each fraction of plasma, buffy coat and RBC was carefully drawn off by Pasteur pipette. Aliquots were taken both for immediate malaria diagnosis (by RDT and microscopy) and for storage to be used later in a qPCR assay. Mononuclear cells from the remaining buffy coat fraction were washed twice in PBSA and resuspended in storage medium of 10% DMSO in foetal bovine serum (FBS) at a concentration of 10^8cells/mL. Cells were stored as MNCs in liquid nitrogen until use. For the peripheral blood (PBMCs), a similar procedure was followed, with the exception of the initial stage of cell dispersion. It might be useful to indicate that both PBMCs and MNCs differ from their respective buffy coats by the fact that buffy coat is equivalent to unwashed MNCs.

3.3 Rapid Diagnosis of *Plasmodium falciparum*

As much as possible, rapid diagnosis of malaria in peripheral blood was carried out within one hour of sample collection. Also, rapid diagnosis was carried out for each Ficoll-stage fraction and tonsil extract, later during the isolation of mononuclear cells from tonsils. The sera of previously established malaria-positive patients were used as positive controls whereas PBSA solution was used as a negative control. Ten microliters of each sample was loaded onto the sample well of Malaria *Plasmodium* falciparum

Rapid Test Device (cassette) (ACON Laboratories, Inc.) and results were read after 15 minutes and recorded as either positive or negative.

3.4 *Plasmodium* Microscopy

Thin and thick blood smears were prepared with heparinized whole blood samples obtained from patients and stained by standard Giemsa staining procedure. Thin and thick smears were each prepared using approximately 10 µl of blood stained with 10% Giemsa stain for 10 minutes. Slides were examined under a light microscope with oil immersion at 100X magnification. Parasitemia was estimated with the thin smears by counting the number of infected RBCs per total number of RBCs for 10 different fields. Although malaria smears normally involve the use of patient peripheral blood, in some cases (in biomedical and forensic research) diagnosis can be made with tissues from the liver, brain, spleen or the placenta (Alunni-Perret *et al*., 2010; Bulmer *et al*., 1993; Peoc'h *et al*., 2000; Uneke, 2007). Encouraged by these reports on the applicability of light microscopy to solid specimens, non-traditional tissues of the tonsils were used to prepare blood smears in this study.

A drop of each Ficoll-stage fraction (plasma, buffy coat, RBC) and their corresponding "tonsil extract" was placed on a clean glass slide. Using a Pasteur pipette, samples were spread gently to cover a circular area. It was ensured that sufficient amount of specimens were used in order to maximize the likelihood detecting parasites in malaria-positive samples. The slides were allowed to dry completely at room temperature. Standard Giemsa staining was then carried out in an identical way as thick blood films

were stained. The slides were examined, using 100X objective with oil immersion under light microscope, and scored as either positive or negative.

3.5 DNA isolation and qPCR assay

DNA was isolated from each set of samples consisting blood spotted on filter paper, PBMCs, MNCs, tonsil extract and ficol-stage fractions: plasma, buffy coat and RBC) using the Quick-gDNA miniprep kit (Zymogen Research Corp.). About 200 ul of whole blood was used to prepare blood spots for each sample by placing blood on filter paper and allowing complete drying at room temperature. Dried blood spots were stored at 4°C until use.

The manufacturer's protocol was followed for all samples except for the blood spots which involved minor modifications to the original protocol. About 5 mm^2 of blood spot was cut out and placed in 1.5 ml Eppendorf tube. One hundred microlitres of tris-EDTA (TE-) buffer (pH 8.0) was added followed by incubation at 85°C for 10 minutes. After 7 minutes of cooling at room temperature, 400 ul of Genomic Buffer was added, vortexed briefly and left to stand for 10 min. The rest of the procedure was similar to the other samples which were treated according to the original protocol. The concentration of DNA was tested using the Nanodrop 2000c spectrophotometer (Fisher Scientific, USA). Custom Taqman Gene Expression Assay (Applied Biosystems) was used to amplify the *Plasmodium falciparum* small subunit ribosomal RNA (ssrRNA) on the BioRad iQ5 PCR machine using primers previously designed by Hermsen and colleagues (2001). Forward primer: GTA ATT GGA ATG ATA GGA ATT TAC AAG GT; reverse

primer: TCA ACT ACG AAC GTT TTA ACT GCA AC. TaqMan MGB probe: 6FAM - TGC CAG CAG CCG CGG TAA TTC – MGBNFQ. Product size expected was 206 bp. The total reaction volume of 20 µl comprised 10 µl 2X iQ Supermix (BioRad); 0.4 µl each of forward primer, reverse primer and probe; 4.8 µl of PCR grade water; and 4 µl of template. For each sample a parallel qPCR was performed for the reference gene of human beta actin (ACTB), with both assays on the same PCR plate. The thermal conditions were 95ºC for 3 minutes (initial hold), followed by 50 identical cycles of 95ºC for 15 seconds denaturation and 60ºC for 1min for both annealing and extension. A single standard curve was generated using control *P. falciparum* (3D7) gDNA (a gift of Dr. Kirk Deitsch, Weil Cornell University, USA, and was used as received) and ACTB gene (BioRad) was performed to determine PCR efficiency for both genes. Results were analysed with the iQ5 Gene Analysis software (Bio-Rad®). Relative quantification was done using the Pfaffl method (Pffafl, 2001).

3.6 Comet Assay

Mononuclear cells (MNCs) isolated from tonsils were examined for chromosomal damage using the Trevigen Comet Assay kit (Trevigen Inc.), in accordance with the manufacturer's instructions for the alkaline unwinding/alkaline electrophoresis protocol. In brief, frozen cells samples were snap-thawed and re-suspended at a ratio of 1:5 in PBS (Ca^{2+}- Mg^{2+}-free). Cells were combined at 1 x 10^5/ml with molten LMAgarose (at 37°C) at a ratio of 1:10 (v/v), and 50 µl of mixture was placed onto the demarcated sample area of the comet slide. Following 10 minutesof incubation at 4°C, slides were immersed in pre-chilled lysis solution and left on ice for alikaline unwinding for 60 minutes at room temperature in the dark. Electophoresis was carried out at 21 volts,

using pre-chilled alkaline electrophoretic buffer for 30 minutes. Slides were immersed for 5 minutes each in water and 70% ethanol respectively, and were dried at 45°C for 5 minutes on a slide dryer. Hundred microlitres of SYBR® Green solution was placed onto each circle of dried agarose and placed in refrigerator for 5 minutes and then dried completely at room temperature in the dark. Images were acquired with the Nikon 80i Upright Research Microscope (software package: NIS Elements) and saved as grey-scaled, TIF format images.

The comet images were analysed later with the CASP Lab software dedicated to analysing comet images. Replicate slides were prepared for each sample, and comets were scored per slide, making a total of at least 12 comets per patient at the minimum. Six analytical parameters were scored. These included head length, tail length, % head DNA, % tail DNA, comet length, and tail moment. An average of all the individual comets representing a patient was computed for all parameters and these quantities were used in the interpretation of the results.

3.7 Statistical Analysis and Data Conversion Procedures

Because there were differences in quantification terms with respect to parasitemia among the various techniques used [microscopy (in percentage), qPCR (in fold change) and RDT (the total number of sub-samples testing positive)], the data were harmonized by first converting the raw, interval data obtained with each method of diagnosis into ordinal data using the "RANK" function of Microsoft Office Excel (2007 edition).

For each of microscopy-based and qPCR-based parasitemia, malaria positive samples were automatically ordered and ranked according to magnitude, with the least

parasitemic sample (in %) corresponding with the rank of 1, in that order up to the most parasitemic sample scoring the highest possible rank in the series. “Overall Rank” was calculated by summing up the two different ranks of microscopy and qPCR for each patient and a ranking was again performed on the “sum of ranks.” Next, these were multiplied by “weights,” defined as the product of the number of methods in which parasite was detected and the number of sub-samples (ranging from whole blood to all tonsil fractions) testing positive, this time including positives by RDT, for a given patient. The data obtained in this manner were put under a column named “parasitemia probability score” and used for the interpretation of results. It must be pointed out that the “parasitemia probability score” so computed was not a percentage-base scale. It simply represented the parasitemia data in a ranked array of values. This method allowed for the logical representation of all considerations relating to parasitemia (inter-technique agreement, frequency of positivity, and parasite load), into a single score.

Similarly, in order to analyze the effect of malaria parasitemia on DNA integrity, patient samples were sorted out into two positivity categories, namely “low-likelihood-of-parasitemia” and “high-likelihood-of-parasitemia” samples. Samples that did not test positive at all by all three methods and those that tested positive by only one method, were together designated as “low-likelihood-of-parasitemia” samples (negative or un-affirmed samples); whereas those that were diagnosed positive by more than one or all three methods were pooled together and designated as high-likelihood-of-parasitemia samples (or affirmed samples). Student t-tests and Spearman's correlation analysis were performed using the Graphpad Prism (v. 5) software.

CHAPTER FOUR

RESULTS

4.1 Pattern of Parasitemia was Unique to Method of Diagnosis and Tissue Type

Of the 22 patient samples examined, 12 (55%) of them were diagnosed positive in whole blood by thin smear alone, with percentage parasitemia ranging from 0.02% to 0.4%. However, only 7 (32% of total number of samples) of these were positive by qPCR, and only one by RDT. For Ficoll-isolated sub-samples (plasma, buffy coat and RBCs), both RDT and qPCR far surpassed microscopy in terms of total number of individual sub-samples testing positive. A total of 19 buffy coat and RBC sub-samples were detected positive in 14 patients by RDT; and a total of 23 positives by qPCR in all sub-sample (including MNCs) representing a total of 16 patients. Thus, overall parasite prevalence rates by method were 55% by microscopy of whole blood, 63% for RDT of tonsils and 73% by qPCR of tonsils (Table 1).

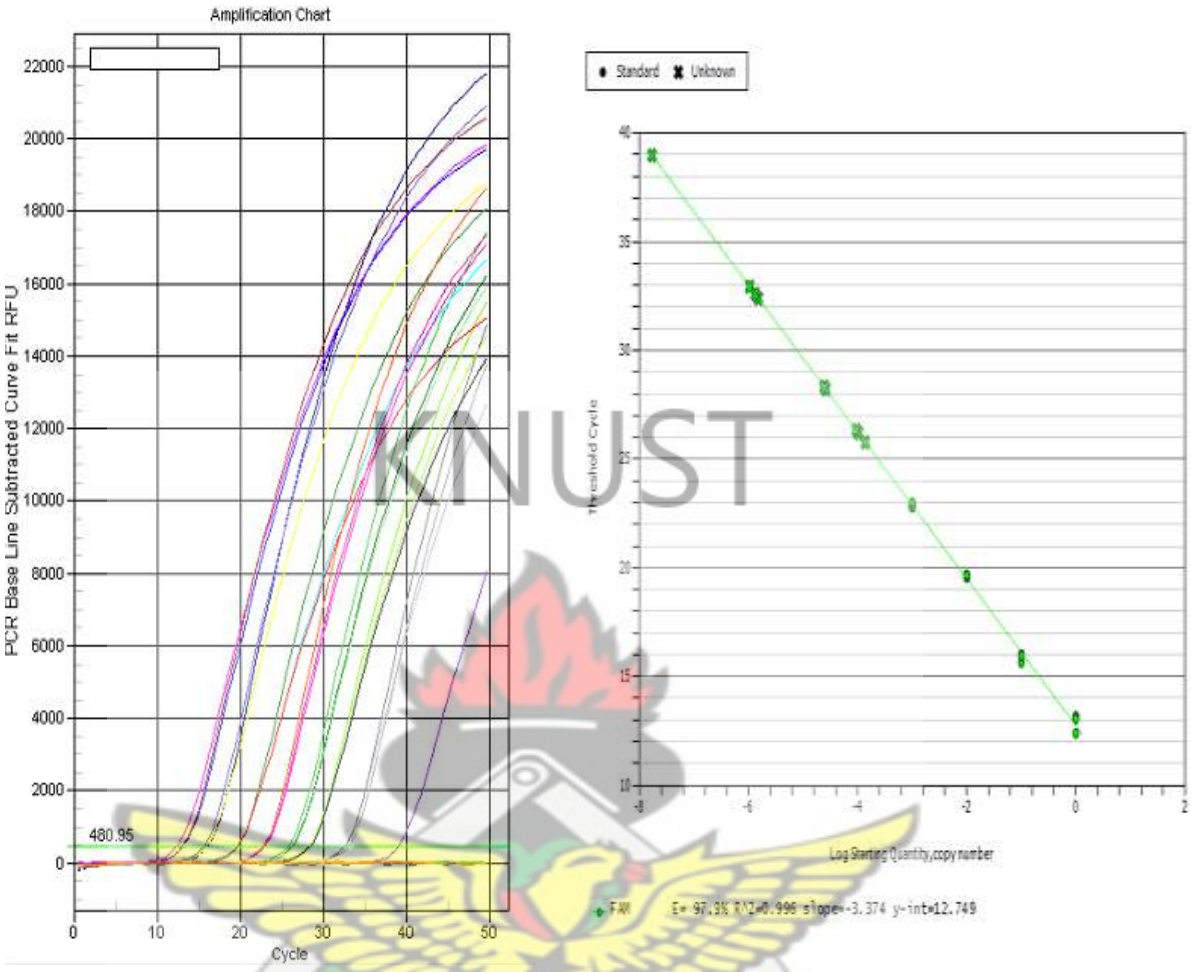

Figure 5: Sample amplification readout and accompanying standard curve for a qPCR experiment.

Source: Original to this work

Table 1. Number of positives based on method of diagnosis for each whole blood and tonsil fraction (Ficoll stage) sub-sample.

	Microscopy(N[a])	RDT(N)	qPCR(N)	Total Positives(N)	No. of Patients(N)	Percent positive (%)
Whole blood	12(22)	1(22)	7(22)	20(66)	12/22	55
Ton. extract	3(22)	0(22)	2(22)	5(66)	4/22	18
Plasma	3(22)	1(22)	4(22)	8(66)	7/22	32
Buffy Coat	2(22)	8(22)	4(22)	14(66)	12/22	55
RBC	1(22)	11(22)	6(22)	18(66)	14/22	63
MNCs	NT[b]	NT	7(22)	7(66)	7/22	32

[a]N: Total number of samples or tonsillar subsamples tested; the sample size in the category

[b]NT: Not tested.

4.2 Levels of Parasitemia in Samples Relative to Period of Sample Collection and Age Category

In order to determine whether or not these data agreed with the malaria transmission pattern widely reported in the literature, a student t-test was performed on the levels of parasitemia accross the six months the samples were collected in. Mean parasitemia probability score was found to be significantly higher in May-June (p-value = 0.0134) compared to the preceding four months of the year (Figure 6A). Similarly, because BL is known to be most common among children aged under 16 years old (Parkin, 1998; Cairo *et al.*, 2003) an analysis was performed to compare the parasitemia level of

patients in this age range with that of their older counterparts. Consistent with expectation, a student t-test revealed a significantly higher level of parasitemia for the 1-16 years age range compared to the older age range (p-value = 0.0257 α=0.05) (Figure 6B).

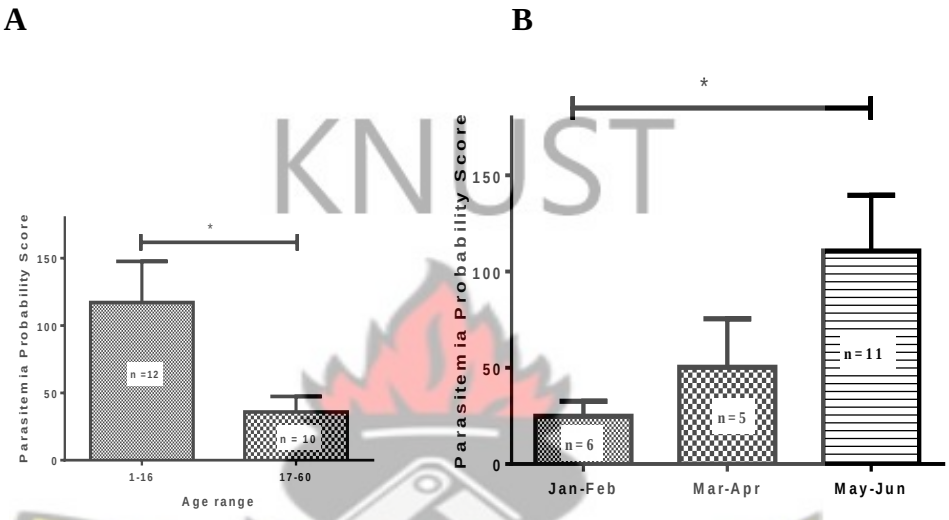

Figure 6: Parasitemia probability score by period of sample collection (A) and Age rage (B). Significant difference (p-value = 0.0134, α=0.05; SEM of Jan-Feb: 24.80 ± 7.794, SEM for May-Jun: 110.6 ± 28.96) was observed between the first quarter of the year and the first two months of the second quarter (May –June). Similarly, significantly different results were shown between children aged 1-16 yrs and persons aged 17-60 (p-value = 0.0257 α=0.05; SEM for (1-16): 117.1 ± 30.47, SEM for (17-60): 35.80 ± 11.55).

4.3 HRP-2 Antigen Concentration in Ficoll Stage Tonsillar Samples

Of the 22 blood samples examined for *P. falciparum* presence, 12 (55%) were diagnosed positive by microscopy of blood and 7 (32%) by qPCR. However, only one sample tested positive with histidine rich protein-2 (HRP-2)-based RDT. On the other hand, upon isolation of tonsillar components by density-gradient centrifugation, 14 (64%) tonsil samples tested positive by RDT and only one plasma sample (which had

tested positive with whole blood) also tested positive. Of these, 57% (8) were diagnosed with the buffy coat fraction alone (Table 2).

4.4 Parasitemia in Ficoll-Centrifuged Whole Blood by RDT

To verify whether these trends could be replicated with whole blood, four (4) whole blood samples all of which had previously tested negative by RDT, were fractionated into the same components as was done with tonsils by density-gradient centrigugation (Ficoll method) (data not shown). Interestingly, with the whole blood, however, all four (4) of the samples tested positive with buffy coat fraction, but not with the RBC fractions. No positive result was recorded by all three methods for peripheral blood mononuclear cells (PBMCs) isolated from peripheral blood.

Table 2: Pattern of positivity between blood and tonsil samples for each patient

Patient no.	Blood			Tonsil			
	Microscopy	RDT	qPCR	Microscopy	RDT	qPCR	Parasitemia Rank
1	X	-	-	-	-	-	Low
2	-	-	-	X	X	X	High
3	-	-	X	X	-	-	High
4	X	-	-	-	-	X	Low
5	-	-	-	-	X	X	Low
6	X	-	-	X	-	X	High
7	X	-	X	-	-	-	High
8	-	-	-	-	-	-	Low
9	X	-	-	X	X	X	High
10	X	-	-	X	X	-	High
11	X	-	-	-	X	-	High
12	-	-	X	X	-	-	High
13	X	-	-	-	-	-	Low
14	X	X	X	-	X	X	High
15	-	-	-	-	X	X	Low
16	X	-	-	-	X	X	High
17	-	-	-	-	X	X	Low
18	-	-	X	-	X	X	High
19	-	-	X	-	-	X	Low
20	-	-	-	-	X	X	Low
21	X	-	X	X	X	X	High
22	X	-	-	-	X	X	High

(X) = Positive test.

(-) = Negative test

4.4 DNA Damage Assessment by Comet Assay

Mononuclear cells (MNCs) isolated from tonsil samples were examined for chromosomal damage by comet assay. DNA damage was estimated in terms of percent tail DNA, tail moment and tail length. Interestingly, assignment of the various DNA damage scores to their corresponding positivity categories revealed a statistically significant difference in the levels of DNA damage between the two different categories of positivity, irrespective of the DNA damage metric used (*p*-value of approximately 0.03 for each of the quantities evaluated (Figure 7A). Similar analysis with percentage parasitemia by thin blood smears also showed a very significant difference between negative or very low parasitemia (<0.05%) and higher parasitemia samples (> 0.1%) (Figure 7B). Spearman correlation analyses revealed a weak, but significant positive correlation between percent parasitemia (thin smears) and each of %Tail DNA, Tail Length and Tail Moment (Spearman's r, P-value: 0.485, 0.0484; 0.4985, 0.0417; 0.5227, 0.0250 respectively). However, no significant difference was observed between male and female genders in terms of DNA damage by all three comet physiologies determined (i.e. % Tail DNA, Tail Lenght and Tail Moment).

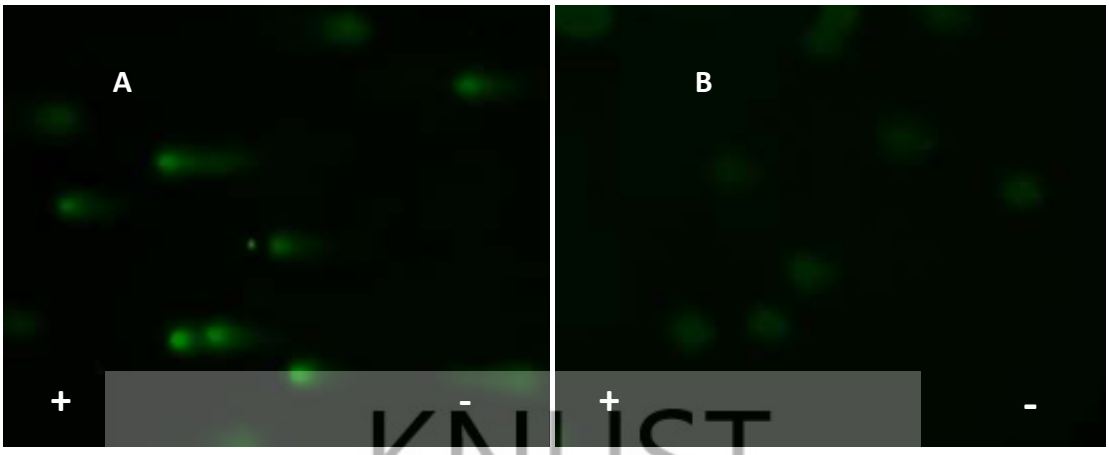

Figure 7 Representative comet images. A. A typical comet image for a high parasitemic patient, showing severe DNA damage evidenced by long comet tails. B. A representative comet image for *Plasmodium falciparum* negative sample scarcely showing any comet tails.

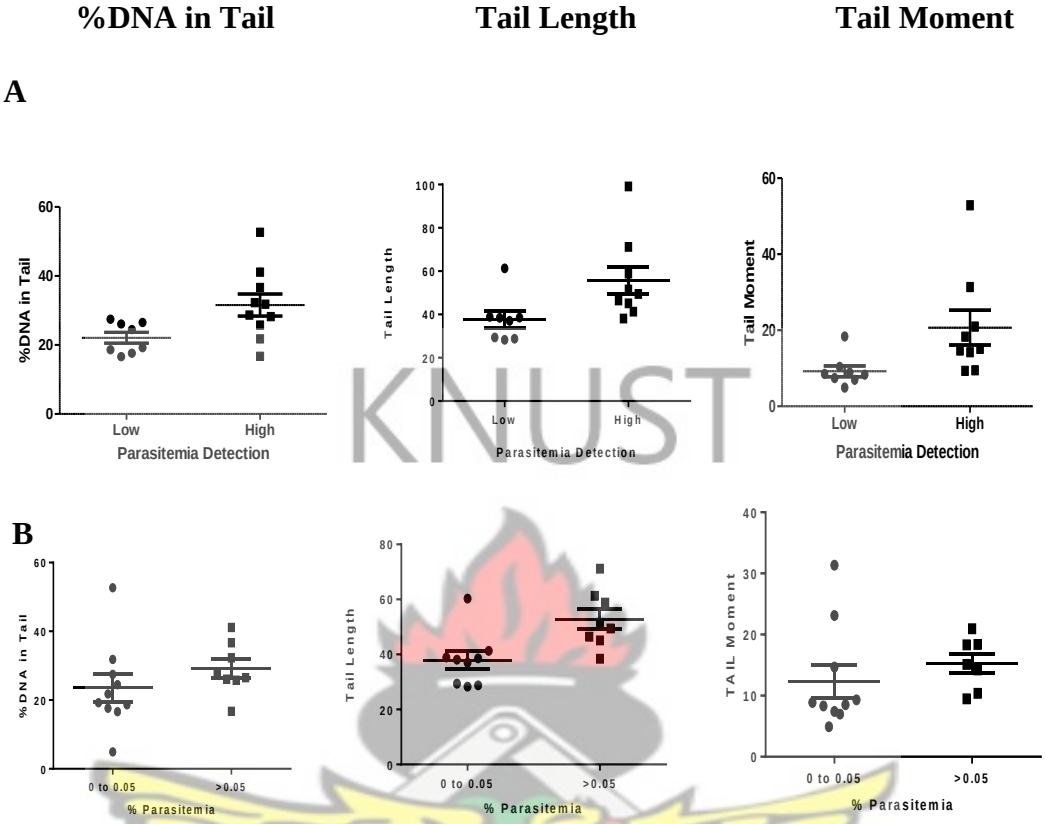

Figure 8: Association between parasitemia and DNA damage. Row **A** compares comet versus *P. falciparum* presence for the quantities of %Tail DNA, Tail Length, and Tail Moment respectively. Whereas row **B** compares comet levels for low and high microscopic parasitemia for the various comet parameters. Analysis of each of the row **A** graphs reveals significant differences between low and high levels of parasite detection (p-values 0.0266, 0.0316 and 0.0389 respectively; α =0.05). However, for the row B graphs, a very significant difference was obtained for only Tail Length (p-value = 0.0083, α =0.05), with no significant difference between low and high parasitemia for each of %DNA in Tail and Tail Moment. (N =22

CHAPTER FIVE

DISCUSSION

Although it was a peripheral interest to this study, the observation that parasite loads were higher during the period between May and June than between January and April, despite the lack of statistical significance (p-value = 0.1962), nonetheless fits the malaria seasonal transmission model expected for the geographical location from which the samples were drawn. This observation was a replication of a recent study conducted in the Kintampo Municipality of the Brong Ahafo Region of Ghana (Owusu-Agyei *et al*., 2009) which shares comparable geographical features with the study area for the present study. It weakly and indirectly supports the notion that high malaria transmission intensity is a factor in the aetiology of eBL.

RDTs have a higher detection threshold than microscopy (approximately 0.2% parasitemia versus 0.0001% parasitemia respectively, (Murray *et al*., 2008); and discordance between microscopy and RDT is found at low parasitemias (<500parasites/microlitre) (Aguilar *et al*., 2012). The concentration of HRP-2 is lowest during early ring stage infections (Desakorn *et al*., 2005). Thus, given that antigen concentrations reached detectable levels suggests that the malaria infections, albeit asymptomatic, were at a later stage where the HRP-2 antigen is produced at increased amounts by the parasite.

The tonsillar equivalent of whole blood was the "tonsil extract" which, even though is supposed to be free from circulatory blood as a result of the repeated rounds of washing

with PBSA, still appeared bloody due to a trace of tonsillar blood. It nonetheless did not come as a surprise that none of the tonsil extracts for the different patients tested positive. This is because it could be that the concentration of any existing parasite antigens may have been further diluted below detection limit by the PBSA used. Thus, it is likely that upon isolating the different cellular components of tonsils (and peripheral blood), the buffy coat and RBC fractions became enriched with parasite antigens thereby raising the concentration beyond the minimum detection limit of the RDT.

Inasmuch as this is a plausible explanation, the question however becomes: But why only with buffy coat and RBC fractions? HRP-2 antigen is a soluble protein (Moody, 2002) which would be expected in the plasma fraction (Desakorn *et al*., 2005; Howard *et al*., 1989; Manning *et al*, 2011). Buffy coat comprises leucocytes and platelets (Visser, 1988). Thus, it is reasonable to suggest that the association between parasite antigen and buffy coat cells/RBCs might not be a mere physical association, as suggested by Zerpa and colleagues (2006).

The fact that parasite DNA was detected by qPCR intonsillar mononuclear cell (MNC) samples obtained from their respective buffy coats, but not in their whole blood suggests an interaction between the mononuclear cells and *P. falciparum* parasites. Mononuclear cells resident in tonsils include monocytes (or macrophages) and lymphocytes (B-cells) that are involved in antigen procession and presentation (Nave *et al*, 2001). This fact may be associated with the detection of parasites in some MNC samples. This inference, however, is valid only to the extent that the qPCR data is valid. It must be

pointed out that there was a problem with the purity of some of the DNA samples (particularly the blood spots), as evidenced by their 260/280nm ratios (data not shown). This fact might have affected the outcome of the qPCR assay.

Another line of evidence that malaria parasites persist even in immune adults is the fact that parasite antigen was detected in over 50% of buffy coat and red blood cells by HRP-2-based RDT. The presence of HRP-2 antigen in buffy coat and red blood cells (although not frequently detected in whole blood in this study) suggests current or recent infection. Expectedly, none of the patients was presenting with clinical malaria at the time of the surgery, nor did any patient believe they had malaria. Indeed, it has been posited that repeated or persistent malaria infection is correlated with eBL risk. Moreover, in a recent report on Ghanaian children showed that, Burkitt's lymphoma risk among a group of Ghanaian children positively correlated with IgG3 antibodies to HRP-2 antigen (Aka, *et al*, 2012). This antigen is *P. falciparum* malaria exposure marker, suggesting that increased exposure to *P. falciparum* evidenced by high HRP-2 antigen load could also be an eBL risk marker among similar populations.

The apparent inconsistency, however, is the observed difference in the pattern of antigen association with buffy coat orRBC between blood and tonsils. Whereas the buffy coat fraction appears to be the sole fraction associated with parasite antigens, both the buffy coat and RBC fractions appear to be associated with parasite antigens in the case of tonsils, although the association with the latter appears to be significantly stronger. However, this could be due to the fact that the smallness of the sample sizes, especially

with blood for which only four (4) patients were studied. However, because the higher positivity rate observed for tonsils was also largely corroborated by the data obtained with peripheral blood fractions, there appears to be an intriguing pattern that demands further investigation.

Although no experimental data in the literature has so far shown the ability of *P. falciparum* to invade lymphocytes, this possibility is nonetheless not remote, given the invasive nature of members of the apicomplexan parasites. *Plasmodium* invasion of lymphocytes, if actual, could be either molecular or organismal or both, and may be sharing similarities with *Yersinia pseudotuberculosis* in its mechanisms for translocating Yops into certain immune cell subtypes (Balada-Llasat *et al*., 2006). Significantly, other Apicomplexan parasites have been shown to interact directly with B-cells. *Theileria* is capable of entering B-cells and driving proliferation via c-myc until the parasite exits the cell (Dessauge *et al*, 2005). *Toxoplasma gondii* secretes a serine-threonine kinase that enters the nucleus of the B-cell in order to subvert the host's immune response (Taylor *et al*, 2006). All of these reports appear to support the notion that *P. falciparum* invasion of B-cells is probable.

These data also appear to affirm that sterile immunity is hardly the case even in adults living in malaria holoendemic areas. A significant proportion of the patients used in this study were adults, most of whom responded a "no" to the question of whether they had taken an anti-malaria drug in the last three months. Although a mere response to such a question cannot be given any diagnostic value, it nevertheless affirmed that patients

used in this study were either "malaria-free" or had mild to asymptomatic malaria. However, Ghana is located within the holoendemic belt of the African continent where *P. falciparum* malaria is stably transmitted, among children and adults alike, throughout the year. Therefore, for malaria parasites to be detected in any form, even in patients who have recently been on malaria chemotherapy, would not have been surprising. Further, intense *P. falciparum* transmission rather than severe malaria is believed to be playing a role in tumourigenesis (Morrow, 1985). This belief is based on the observation that in places where *P. falciparum* transmission is seasonal rather than stable, the chromosomal translocation is always different than that for eBL (Magrath *et al*, 1992). Significantly, these data show a positive correlation between parasitemia and DNA damage. This observation fits the hypothesis for this study that malaria directly induces chromosomal damage. This study did not look into the nature of the damage in order to gain insights into whether or not the typical eBL translocation was present. It is likely as has been advanced before, that different factors have conspired to produce the damage observed in this study. Nevertheless, a report on an infection equally potent for generating free radicals offers an experimental demonstration to the potency of ROS in oncogenesis. Machado and others (2009) report that in mice chronically infected with *Helicobacter pylori*, which causes gastric cancer, a decrease in mismatch repair gene expression was observed along with induction of DNA damage through increase in ROS generation, similar to the levels observed with damage by hydrogen peroxide (H_2O_2) a known DNA damaging chemical.

Whiles this was completely expected and most likely valid, it must be noted that the inherent weakness of this assay could have contributed to a possible bias. Currently, there is no consensus on standard statistical methods for the analysis of comet data (Tice *et al*., 2000). This fact, coupled with the lack of malaria-free controls, and the fact that the sample size was small, could have affected the overall picture of the comet data.

Taken together, this study has yielded data that encourage the notion that *P. falciparum* malaria can induce chromosomal breakage that sets the stage for oncogenesis. Also, these results suggest that detecting parasite in tonsils is actually more probable compared to whole peripheral blood that has been the tissue used traditionally in the diagnosis of malaria. For the same patient whole blood sample that tested negative by an RDT, when fractionated into its cellular components by density-gradient centrifugation, detection of parasite antigens in either the RBCs or buffy coat by a strip from the same lot of RDTs, was often enhanced. These data suggest that parasite antigen load is strongly associated with mononuclear (mainly leukocytes) and RBCs both of which are cell types that interact directly with malaria parasites during immune and pathogenic reactions inside the human host. These observations need to be investigated further, in order to afford a better understanding of the cancer-causing potential of malaria infection.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

This study has shown that *P. falciparum* resides in the tonsils. Additionally, it has been demonstrated that *P. falciparum* parasitemia also correlates positively with DNA damage in tonsillar mononuclear cells. Given that genomic instability is the precondition for cancer progression, the correlation observed between malaria parasitemia and DNA damage may be supporting the idea of a direct causal link between malaria and cancer, even in eBL. However, it is important to consider that correlation may not entail causation, particularly as none of the study participants was known to be having any type of cancer. These data would have been more enlightening if the specific translocation could have been found. These results nonetheless strengthen the burgeoning notion that malaria could directly cause cancer. Additionally, it was found that parasite diagnosis by rapid diagnosis (RDT) is improved over whole blood samples when RBC and/or buffy coat fractions obtained after density-gradient centrifugation of patient blood, are used, although this result is very preliminary, and requires further validation.

6.2 Recommendations

The following recommendations are offered as ways of gaining deeper understanding of the mutagenicity of *P. falciparum* and ways of developing practical applications of these findings.

1. Future studies need to look at the cytogenetics and the molecular biology of tonsillar mononuclear cells isolated from *in vitro* malaria-stimulated MNCs to elucidate the molecular signatures of the DNA damage attributable to malaria, and to verify whether or not the translocation is present.
2. Also, it may be worthwhile to investigate the association between holoendemic malaria and tonsillitis, as the two diseases appear to share comparable epidemiological features. The same season in which the largest number of tonsils was collected was the same period (May-June) parasitemia was found to be highest. A study based on larger sample size is recommended in order to verify the association suggested by these results.

3. In order to gain a holistic understanding of any role that malaria might be playing in any cancer, a multidisciplinary approach is required. It is believed that an understanding of the complex parasite-host interactions in the course of an infection with oncogenic microbes, would leverage an understanding of a number of related conditions including ageing and degenerative disorders (Blaser, 2008). The paucity of information currently available in the literature or the lack of any understanding on the relationship between malaria and genetic instability or cancer (Lehrer, 2010), bears testimony to the impression that the

parasite has long been overlooked or at least underestimated by researchers in terms of its cancer-causing potential. Apart from the malaria-EBV linkage in endemic Burkitt's lymphoma, in which significant inroads have already been made, research interest in malaria as a potential, independent causative factor in the aetiology of relevant cancers is only now emerging.

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APPENDIX

8th April, 2013

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CONSENT FORM

INFORMATION SHEET

Title of the research:

Characterization and Analysis of Lyphomas with Special Focus on the Pathogenesis of Endemic Burkitt's Lymphoma

Name(s) and affiliation(s) of researcher(s) of applicant(s):
Dr. Karen Duca of KNUST with co-principal investigators and key personnel from KATH/SMS KNUST, NMIMR, Tufts University, Boston, MA (USA), and Virginia Tech, Blacksburg, VA (USA)

Purpose(s) of research:

The main objective of this project is to characterize lymphomas in Ghanaians using more sensitive and reliable molecular genetic and immunohistochemical methods, with a special focus on gaining a mechanistic understanding of the early events that trigger endemic Burkitt's Lymphoma.

Procedure of the research, what shall be required of each participant and approximate total number of participants that would be involved in the research:

Participants who decide to enroll will donate a tumor biopsy sample, bone marrow sample, and/or blood sample. With the exception of the blood, the tissue donations form part of the usual diagnosis and staging of the lymphoma and would be done as part of the normal course of diagnosis. After the tissue donation, no further participation is required. For the non-cancer tonsillar tissue samples, routine tonsillectomy patients may elect to donate their tonsils for scientific research, as well as a small amount of blood (no more than 10 ml). A maximum of 200 BL patients will be enrolled, if possible.

Risk(s) & Benefits: DIRECT

Lymphoma patients: The clinical team will receive feedback with regard to the type of lymphoma based on analysis of surface markers by flow cytometry. This will assist them in clincial management. In the longer term, those same samples that have the genetic lesion indicative of eBL will be identified and the information conveyed to the clinicians for improved future diagnosis. Their donation also contributes to the advancement of medical science and West African epidemiology.
Control Patients: There are no direct benefits to the control patients. Their donation contributes purely to the advancement of medical science and West African epidemiology.

Benefit(s): INDIRECT

Indirect benefits in this proposal are mainly long term and relate to future patients and better treatments resulting from increased understanding of the process of eBL development. Moreover, by analysing the tumor samples a clearer picture of lymphoma epidemiology in Ghana will emerge that will assist physicians in associating clinical presentations with the underlying pathology. Results of the study may also generate new ideas and spawn additional research projects.

Confidentiality:

All information collected in this study will be given code numbers and no name will be associated with the patient during the analysis of samples. (A master list will be deposited with KATH administration in order to give clinicians feedback on patient care and in case there is a need to contact patients about a future study. No member of the research team will have access to this list during the course of the project.) The patient's data cannot be linked to him in any way nor will any identifier be used in any publication or reports from this study. As part of our responsibility to conduct this research properly, various officials from government or international regulatory bodies may request access to raw data. In most cases, this will involve only the de-identified data, but may involve examination of our master list as well to validate compliance.

Voluntariness:

Your participation in this research is entirely voluntary and may be terminated at any time without penalty. Once the tissue samples have been taken, your active participation is over. If desired, you will receive a copy of all research publications resulting from your donation. Please give your contact information to your clinician if you would like to pursue this option.

Alternatives to participation:

If you choose not to participate, this will not affect your treatment in this hospital in any way. Any questions you may have about the scientific aspects of the study will be gladly answered by either of the post-graduate students involved in the project or your clinician. Their contact information is given at the end of this form.

Consequences of participants' decision to withdraw from research and procedure for orderly termination of participation:

You can choose to withdraw from the research at anytime. Please note that some of the information that has been obtained about you before you chose to withdraw may have been modified or used in reports and publications. These cannot be removed anymore. However the researchers promise to make good faith effort to comply with your wishes in the future as much as is practicable and within the bounds of scientific integrity.

Statement of person obtaining consent:

I have fully explained this research to _____ and have given sufficient information, including about risks and benefits, to make an informed decision.

DATE: _____ SIGNATURE: _____

NAME: _____

Statement of person giving consent:
I have read the description of the research or have had it translated into language I understand. I have also talked it over with the interviewer to my satisfaction. I understand that my participation is voluntary. I know enough about the purpose, methods, risks and benefits of the research study to judge that I want to take part in it. I understand that I may freely stop being part of this study at any time. I have received a copy of this consent form and additional information sheet to keep for myself.

DATE: _____ SIGNATURE/THUMB PRINT: _____

WITNESS' SIGNATURE (if applicable): _____

WITNESS' NAME (if applicable): _____

Contact information for the graduate students involved in this research is given below. You are free to contact them if you have any questions relating to the scientific aspects of this project. For clinical and treatment questions, you are referred to your treating physician.

Mr. Charles Torgbor
Dept. of Biochemistry and Biotechnology
KNUST, Kumasi

Mobile: 0242245198
E-mail: charles.torgbor@gmail.com

Mr. Godfred Futagbi
Dept. of Zoology
University of Ghana, Legon

Mobile: 0244012416
E-mail: fgodfred@yahoo.com