KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY

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DEPARTMENT OF BIOCHEMISTRY & BIOTECHNOLOGY

Prevalence of Chloroquine Resistance in Urban Versus Rural Communities near Kumasi, Ghana

THIS THESIS IS PRESENTED TO THE DEPARTMENT OF BIOCHEMISTRY & BIOTECHNOLOGY IN PARTIAL FULFILLMENT FOR THE REQUIREMENT FOR THE AWARD OF MASTER OF SCIENCE IN BIOTECHNOLOGY

BY

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DECLARATION

"I declare that I have wholly undertaken this study reported therein under the supervision of Dr. Mohamed Mutocheluh and that except portions where references have been duly cited, this Dissertation is the outcome of my research".

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ABSTRACT

The fight against malaria, despite recent strides, is still not fully won. This fact can be attributed to ineffective diagnostic tools for rapid and accurate diagnosis, use of older drugs against which the parasite has developed resistance, and failure to rigorously practice prophylactic measures. Chloroquine was once the preferred drug to treat malaria in Ghana; about 8 years ago the WHO recommended a switch to artemisinin-based combination therapy (ACTs). Thus, sensitivity to chloroquine might be expected to return. One hypothesis is that the sensitivity of *Plasmodium falciparum* to chloroquine might be higher in rural areas with less access to chloroquine, as compared to urban centers with easy access to it as well as other treatment options that work on the same mechanism. An alternative hypothesis is that chloroquine circulates more widely in the rural sector among licensed chemical sellers (LCS) and informal vendor networks. This study aimed: A) to determine the accuracy of routine thick smear diagnosis under usual working conditions as compared with nested PCR and B) to ascertain the prevalence of chloroquine resistance in central, urban Kumasi (KATH, 100 samples), peri-urban Kumasi (KNUST-Hospital, 108 samples) and a more rural area location (Nkwantakese and Pampatea, about 20 km north of Kumasi, 203 samples). A total of 403 thick smears and blood spots with an initial malaria diagnosis made by trained microscopists were collected in the three locations from December, 2012 to January, 2013. Genomic DNA was extracted from the spots and a segment of the *Plasmodium falciparum* chloroquine resistance transporter gene (*pfcrt*) was amplified. The amplicons were digested with the restriction enzyme Xap1 to determine the presence of the K76T mutation in *pfcrt* gene of malaria parasites. Out of 403 samples, a total of 27 (7%) were diagnosed positive by microscopy as compared to 39 (10%) by PCR, with 376 (93%) and 364 (91%) identified negative by microscopy and PCR, respectively. Only 13 smear positive samples were confirmed by PCR and 26 smears were falsely diagnosed negative by microscopy. Although there were far too few positive malaria tests for statistical significance, chloroquine resistance in the *P. falciparum* population did not decrease as one moved from the urban centres (KATH and KNUST-Hospital) towards the more rural setting (Nkwantakese and Pampatea). A resistance of 87% (20 out 23) was observed for the urban and peri-urban centres together, whereas, a higher prevalence of 94% (15 out of 16) was noted in the rural areas. This preliminary result suggests that chloroquine use might actually be higher in the villages than the urban area. More work is required to validate this very preliminary trend.

DEDICATION

This work is dedicated to my family and my wife, Maame Yaa Baidoe-Ansah

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Basically, it is ideal to always show appreciation to any assistance received from others, hence, I am highly grateful first and foremost to the Almighty GOD for His incomprehensible grace and favour throughout my pursuit of this degree.

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CHAPTER ONE

1.0 INTRODUCTION

2.0 Background and Significance

To effectively treat malaria, it is critical to exigently identify infected individuals in time, thus requiring accurate diagnostics. Malaria is typically diagnosed by either microscopic examination of blood using Giemsa-stained thick or thin blood films, or with antigen-based diagnostic tests commonly known as Rapid Diagnostic Test (RDT). Not only are the aforementioned techniques valid, but now, modern molecular-based tests, polymerase chain reaction (PCR) technique detects parasitic DNA, albeit not widely used in malaria-endemic areas due to their high costs and test complexity (Perkins and Bell, 2008). Comparing the three said methodologies, PCR is the most sensitive with a range of ≥5 parasites/µl of blood followed by 50 parasites/µl of blood for microscopy, with RDT having a detection limit of >100 parasites/ µl of blood (Bell, 2004; Ratnawati *et al*., 2008). As a result of the different detection limits, utilization of the above known methods of diagnosing malaria infection are dependent on the endemicity of the disease (Golassa *et al*., 2013).

After accurate identification of malaria infected individuals, the recommended mode of treatment is chemotherapy, though, it can also be prevented by chemoprophylaxis. Initial chemotherapies, the mono-therapies - chloroquine and artesunate to name a few, were very effective from the word go, but later battled with resistance in malaria parasites, especially *Plasmodium falciparum*, subjecting them to be permutated. This culminated in changes in malaria treatment policies in some endemic nations from mono-therapy to combination-based therapy (WHO, 2009). On the market are artemisinin in combination with other anti-malarials like amodiaquine, lumefantrine, mefloquine or sulfadoxine/pyrimethamine

(WHO, 2010), dihydroartemisinin, and piperaquine (Keating, 2012). Worldwide reports on current malaria incidence and burden indicate a drastic decrease of about 24%, including endemic regions like sub-Saharan Africa (WHO, 2013). This outcome has been associated to the high treatment efficacy of 90% observed for artemisinin-based combination therapies, (ACT's) 90% for treating uncomplicated malaria (Howitt *et al*., 2012).

Nonetheless, the treatment of malaria has been faced with several challenges like drug resistant parasites and non-compliance to stipulated treatment method, whereas, the latter has a part to play in the development of the former. Drug resistance in malaria parasites has accounted for the treatment failures associated with most anti-malaria drug therapies, which explains why the highly esteemed treatment of malaria, with ACT's, has about a 90% treatment success (Howitt *et al*., 2012). At the moment, previous surveillance confirms the emergence of resistance in *P. falciparum* towards ACT's, for instance in Cambodia in Southeast Asia (Newton *et al*., 2006; O'Brien *et al*., 2011). The emergence of resistant parasites to ACT's is very alarming as it predisposes this known effective treatment method to the same fate as chloroquine, a once effective chemotherapeutic agent against *P. falciparum* malaria.

Chloroquine lost the spot as the first line malaria treatment after *P. falciparum* parasites resistant to the drug originated from Eastern Asia, to more endemic impoverished locations like sub-Saharan Africa (Ridley, 2002). This outburst led to numerous studies to ascertain the cause and possibly characterize the new variant parasite. Fortunately, false diagnoses, continual usage of poor quality drugs and patient's attitude towards treatment plans were confirmed as prime causes of chloroquine resistance in *P. falciparum*. Further studies elucidated the genetic, physiological and pharmacological aspects of chloroquine resistant

parasites, from which the digestive vacuole was identified (Verdier *et al*., 1985; Krogstad *et al*., 1987; Saliba *et al*., 1998). This was followed by insights into the genetic variation between sensitive and resistance strains. A point mutation at the 76th position of the *P. falciparum* chloroquine resistant transporter (*pfcrt*) gene was identified to account for the resistance (Bray *et al*., 2005). Also, the multidrug resistance 1 (*pfmdr1*) genes, although less prominent compared to *pfcrt* gene, were identified as contributors to chloroquine resistance (Setthaudom *et al*., 2011).

In Africa, cases of chloroquine resistance to *P. falciparum* emerged in 1978 initially in the eastern part of the continent (Sa *et al*., 2009). This prompted the World Health Organization to recommend a change in drug policies from mono-therapy to combination therapy, which finally culminated in the use of ACTs, to which some African countries like Malawi and Kenya came on board as early as the 90's (Davis *et al*., 2005). Interestingly, follow up studies in some of these countries years after the switch in drug policies documented return of sensitivity of malaria parasites towards chloroquine (Kebede *et al*., 2014; Kublin *et al*., 2003; Mwai *et al*., 2009). However, in Ghana, high incidence of chloroquine resistance has been observed before and after the nationwide switch to artemisinin-based combination therapies (Duah *et al*., 2007; Abruquah *et al*., 2010), with these findings validated by reports of the existence of chloroquine drugs on the market (Asare *et al*., 2013), despite being ostracized in the country as far back in 2004 (Duah *et al*., 2007). Meanwhile, these studies were carried out in some selected communities with easy access to health facilities and chemical shops within the urban umbrella of the country. This study goes a step further to elucidate the influence of distance on the incidence of chloroquine resistance, as one moves from the urban centres to a more rural setting where there is little or no access to well-stocked chemical shops.

1.1 Study objective

This study sought to determine the prevalence of chloroquine resistant *P. falciparum* in the Ashanti region between two different sites (rural and urban communities). Specifically, three aims were engaged in the study.

1.1.1 Specific objectives

1. Determination of the level of resistance of *P. falciparum* to chloroquine in Ashanti region.

2. Determination of the accuracy of malaria diagnosis by microscopy using nested-PCR as reference.

3. Measurement of the difference in sensitivity of *P. falciparum* to chloroquine between rural and urban areas.

1.2 Study hypothesis

A correlation exists between the local availability and use of chloroquine and resistance to that drug in *P. falciparum*. This means there could be a variation in resistance between Kumasi city and outlying remote areas where drug availability is lower. However, a great number of chloroquine sensitive *P. falciparum* parasites are expected in locations having limited or no access to well stocked-pharmaceutical shops. That being said, it is also possible that Licensed Chemical Sellers (LCS) in the rural areas may prefer to stock chloroquine, as opposed to the more effective artemisinin-based combination therapies (ACT's) due to the lower costs. Thus, more resistance might be observed than in Kumasi city. This study represents an attempt to determine if there is any difference in resistance levels as a function of distance from Kumasi city centre.

1.3 **Problem statement**

Although studies suggest that rapid and accurate diagnosis of malaria positively affects the reduction of malaria morbidity and mortality (WHO, 2013), limitations associated with current malaria diagnostic methods render the overall objective unachievable. Diagnostic errors have been implicated as a contributor to the development and continual transmission of drug resistant parasites. Therefore, resistant strains persist in the environment and treatment failures become increasingly common.

In 1993, Malawi was the first malaria endemic country in Africa to sign onto the new drug policy, the artemisinin-based combination therapies (ACT's) (Bloland *et al.*, 1993) and subsequent studies confirmed a change in chloroquine sensitivity in malaria parasites. Initially about 85% resistance was observed coupled with approximately 50% treatment

failures (Kublin *et al*., 2003) and subsequently changed to ACT's with sensitivity returning a decade later (Laufer *et al*., 2006). In addition, Kenya instituted similar policies as Malawi and reported similar outcomes (Mwai *et al*., 2009). However, this was not the case with Ghana, which switched to ACT's in 2005 (Alam *et al*., 2011). In the early 1980's, Ofori-Adjei and colleagues reported on chloroquine resistance for the first time in Ghana (Ofori-Adjei *et al*., 1984), which was low, but some years later, studies confirmed a drastic increase in resistance at the southern part with lower percentages moving up (Neequaye *et al*., 1986). However, conditions in the northern part worsened by 1998, with 57% prevalence of resistant strains (Ehrhardt *et al*., 2002). This prompted the change in anti-malaria drug policies in 2004, which took effect a year later to the ACT's (Koram *et al*., 2005).

Nearly ten years later discouraging results are emerging after continuous monitoring in selected endemic sites in Ghana. For instance, a study conducted by Abruquah and colleagues in 2008 from 140 children randomly selected at the Komfo Anokye Teaching Hospital (KATH) in Kumasi, observed the T76K mutation in 124 samples corresponding to approximately 88% prevalence (Abruquah *et al*., 2010). In addition, a study conducted at Kwame Nkrumah University of Science and Technology (KNUST), 8.9km west from KATH, four years later, also observed 38 resistant *P. falciparum* out of 39 positive samples equalling to about 97% resistance (Azumah *et al*., 2012). A more recent study also identified the T76K mutation in 246 cases out of 1,318 subjects (Afoakwah *et al*., 2013). These results have suggested increasing prevalence of chloroquine resistance which can be attributed to persistent usage of chloroquine.

Although reductions in malaria as compared to the past have been noted, the need to accurately and rapidly diagnose malaria and treat it appropriately remains. This will help to further decrease the annual incidence and prevent the development of resistance to ACT drugs.

However, chloroquine has been identified on the drug market by Asare *et al*., (2013) despite being outlawed for mono-therapy. This makes control and reduction of resistant parasites to Chloroquine a daunting task. Definitely, getting chloroquine completely out of the system may result in the return of sensitivity to chloroquine in resistant parasites, as was observed in Malawi. This calls for the in-depth study to elucidate possible factors contributing to the rather increasing prevalence of resistant parasites in studied populations in Ghana and recommend possible ways forward.

1.4 **Study justification**

The idea of the Ghana Health Service to follow in the strides of Malawi and Kenya (Johnson *et al*., 2004), in the use of Chloroquine as a first line response to malaria (O'Brien *et al*., 2011; Ariey *et al*., 2014) is yet not attainable. Return of chloroquine sensitivity might seem unrealistic, however, studies have confirmed substantial mutation in resistant *P. falciparum* that later became susceptible to chloroquine treatment (Johnson *et al*., 2004; Wellems, 2004).

However, as it stands, in years past, there is no promising data regarding the return of chloroquine sensitivity in Ghana (Abruquah *et al*., 2010; Azumah *et al*., 2012), though, they were all gathered from urban communities, where there is easy access to

chemotherapy as compared to more rural settings with no easy access, thus resulting mostly in the use of herbal preparations.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Epidemiology and Disease Burden of Malaria

Malaria infection continues to drain resources from governmental and non-governmental agencies, with current global malaria incidence experiencing about 75% decline in newly reported cases with about 43 African countries reporting such results (WHO, 2013; Gething *et al*., 2014). Malaria infection is currently known to be caused by five (5) *Plasmodium* species namely; *P. falciparum*, *P. malariae*, *P. ovale. P. vivax* and *P. knowlsi* (Berghout and Gros, 2007). Most malaria cases in Ghana are caused by *P. falciparum*, accounting for 90% of all cases with 9% for *P. malariae* and *P. ovale* responsible for the remaining 1% (Appawu *et al*., 2001; Yawson *et al*., 2004). Malaria infection from the remaining species, *P. vivax* and *P. knowlsi*, do not occur in Ghana (Lindsay and Hutchinson, 2006).

Tools like chemotherapeutic agents, more refined diagnostics, insecticides against vectors, and understanding of the seasonal impact of malaria, have all been tried to effectively control the high malaria morbidity and mortality. The incidence of this disease is high enough to trouble both infected individuals and the economic status of burdened nations, although recent data confirms a decrease in annual malaria cases and mortalities. It is estimated that about 90% of malaria mortalities occur in Africa, especially, the southern part of the Sahara which is known to be endemic to the disease (WHO, 2009). However, compared to the former disease burden (WHO, 2009), seemingly, a decrease in annual malaria incidence and mortalities has been observed, which brings smiles to the faces of those strongly advocating for the complete eradication of malaria (WHO, 2013).

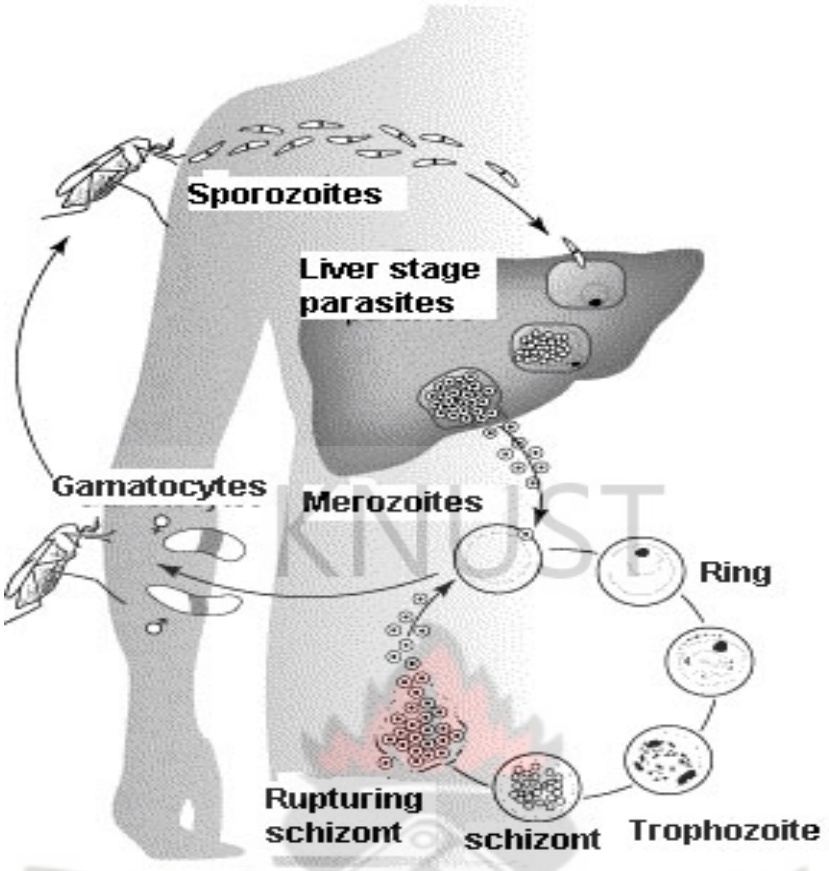

Figure 2.1: Life Cycle of Malaria Parasites

The diagram gives an overview of the mechanism under which a malaria parasite completes its life cycle (Joao, 2008). The parasite requires two hosts, mosquito and human, with the latter serving as the definite host. Malaria parasites initially mature and reproduce in the liver of the definite host after being delivered through the saliva of the female anopheles mosquito, to invade red blood cells. Malaria infection comes with symptoms like fever and headaches (Joao, 2008).

In Africa, pregnant women and children from the ages of 1-4 years are the most vulnerable due to the stable mode of transmission of the disease (WHO, 2002). The early years of a child's life is the most dangerous due to inadequate clinical immunity, therefore, most infant malaria related mortalities occur in the first year or two (WHO, 2002). Adult women on the other hand have a higher level of immunity but this is impaired during pregnancy, which accounts for the

increased malaria susceptibility (WHO, 2004). Referencing worldwide annual malaria deaths, there is a high rate of fatality by malaria which can be due to the late presentation, inadequate management and unavailability or stock-outs of effective drugs in impoverished continents (WHO, 2004). Also the presence of resistant parasites to any wrong anti-malaria drug predisposes the public to a high extent of treatment failures which eventually culminate in high morbidity and mortality rates (Trape, 2001). Meanwhile, misdiagnosis can lead to the burden of malaria since many of the health facilities in impoverished nations like Ghana lack the laboratory capacity which makes it difficult to distinguish malaria from other infectious diseases (Adams *et al*., 2004).

Malaria incidence in the western part of Africa, especially Ghana, has been observed to be high during the rainy season as there is abundance of the vector, female anopheles mosquitoes whereas the reverse is observed in the dry season (Jawara *et al*., 2008). During the rainy season, the preferred sites by the vectors for proliferation are water collected in pools, potholes and excavations, thus, efforts to impede the stagnation of water eventually prevents mosquito bite and subsequently reduce transmission. However, this is not the case during the dry season as the environment is rid of stagnant water and reduced mosquito populations (Adjei, 2010).

Moreover, varying transmission levels occur between urban and rural communities, with the former known to record low transmission levels than moving further towards the less populated communities withpoor drainage system (Lines *et al*., 1994). Another factor accounting for the difference in malaria transmission is the difference in climate (Robert *et al*., 2003) as the remote communities record high annual rainfall than the urban settings, there are more breeding sites for the mosquitoes and eventually more malaria cases. In 2012, Ankomah observed some

factors that can effectively interfere with malaria transmission, including the use of treated mosquito nets and insecticide sprays (Ankomah *et al*., 2012).

Transmission intensity is the frequency with which an individual is bitten by an infected vector, female anopheline mosquito. It is known to affect certain aspects of malaria epidemiology, especially, the prevalence, incidence and the mortality of the disease (Hay *et al*., 2008), which are useful in the determination of the endemicity of the disease at hand. In 2010, using the transmission intensity method, the highest proportion of malaria clinical cases in Ghana were observed in the Upper West Region, with the least transmission located in the southern part especially, Greater Accra region (Gething *et al*., 2010). A possible explanation for the high endemicity of malaria infection in the northern sector (Figure 2) can be the poor drainage system coupled with the limited health care providers, amidst the dryness of location.

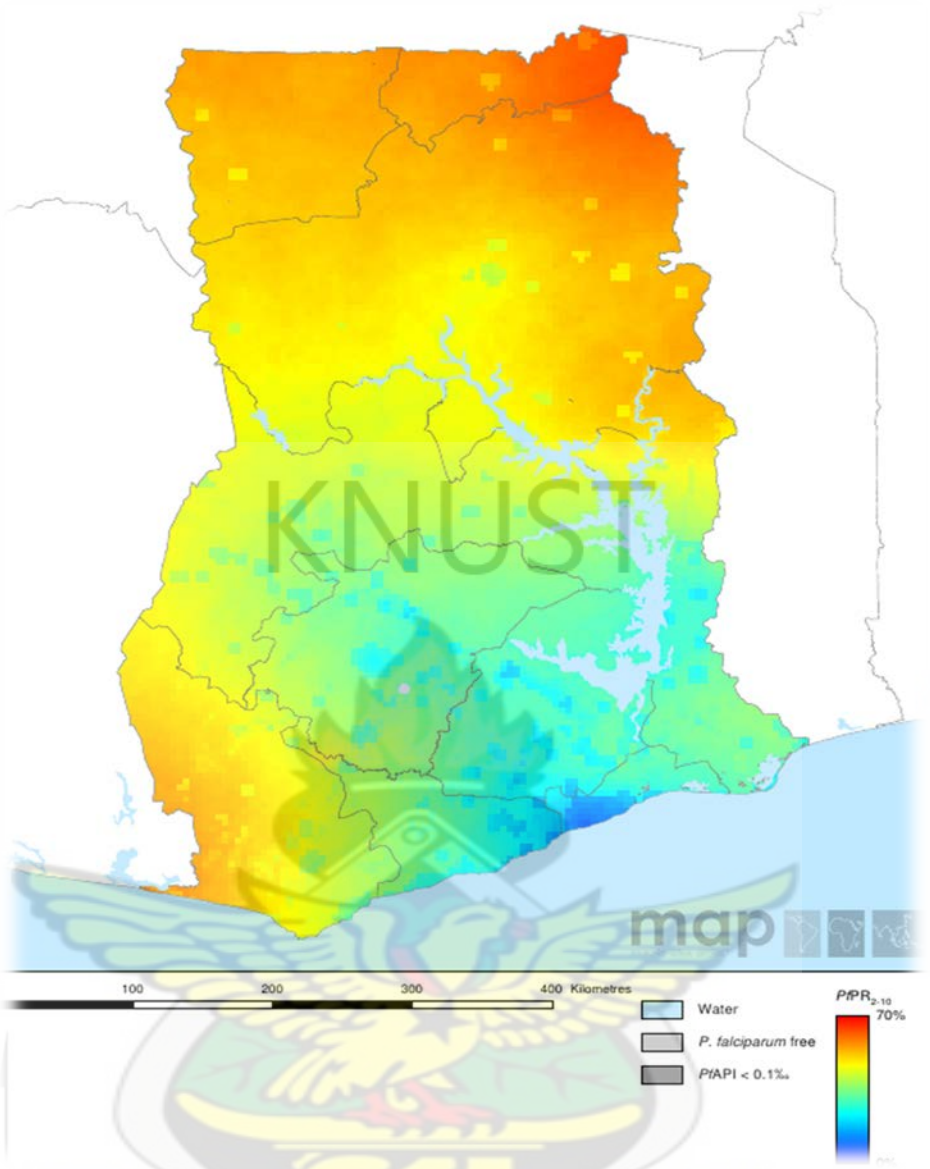

Figure 2.2: Map of *P. falciparum* Endemicity in Ghana

This figure represents the distribution and endemicity of malaria parasites in Ghana for the year 2010 (Gething *et al*., 2010). Data being used for this map were sampled from clinical cases documented in almost all health facilities in the country. According to the above map, the northern part of the country experiences high abundance of malaria cases, (indicated area in red). However, as one moves further down to the coastal areas there is a substantial decrease in the level of endemicity of malaria parasites, specifically *P. falciparum* (Gething *et al*., 2010).

2.2 Malaria Diagnosis

Currently, three diagnostic methodologies are in common use to detect malaria infected individuals, viz., microscopy, rapid diagnostic test (RDT) and polymerase chain reaction (PCR). Microscopy and RDT are the most common methods in Ghana with microscopy being the most employed. These three methodologies have varying accuracy, sensitivity, and cost which is summarized in the table below.

Table 2.1 Pros and Cons of the 3-Methods of malaria diagnosis

	Microscopy	**PCR**	**RDT**
Sensitivity	Thick: 5-10 per µl Thin: 100-200 per µl	0.4 parasites per µl	~100 parasites per µl
Cost	~ 1GH₵ per test	~15 GH₵ per test	~3 GH₵ per test
Time used	~ 40 min	~ 1 day	~ 3 min
Equipment	Need microscope	Need equipment	Nothing
Expertise	Trained expertise	Trained expertise	No expertise
Errors	High, in both directions	With controls, few	Embedded controls

2.2.1 Microscopy

This is the standard for routine diagnosis of malaria in Ghana. In tropical countries, microscope is almost present in every medical laboratory for clinical diagnosis of malaria due to its unique sensitivity and specificity (50 parasites/µl of blood for microscopy). In addition, this technique is suitable due to its short time for examination (about 60 minutes) and simplicity of its operation (Bain *et al*., 1997), lower cost, ability to determine specific species and quantitation, and determine the progress of antimalarial drugs (Bell and Perkins, 2008). Microscopy is also recommended as the standard reference in assessing the efficacy of any new diagnostic methodology (Wongsrichanalai *et al*., 2007).

There are two ways in which a microscope slide for diagnosis can be prepared; thick and thin smears. The thick smears are also used to determine the number of malaria parasites (quantitative) (Sherman, 2009). One can be more quantitative with the thin smear with only a monolayer of cells. This technique requires patients' blood (about 6µl) spotted on a glass slide, air dried and stained with approximately 5% Giemsa dye, which is prepared using alcohol as the buffer (Cheesbrough, 2005). The smear is then observed under the microscope and the presence of malaria parasites are determined using the plus scale system (0-4+) depending on the amount of parasite per viewing field. Negative (-) or 0 represents the absence of malaria parasites in 100 high-power fields whereas + sign denotes the presence of malaria parasites, thus positive. A plus of one (1+) represents the presence of about 1-10 parasites per 100 high-power field. The highest parasite density is 15abelled ++++ (4+) which stands for beyond 10 parasites per each high-power field (Cheesbrough, 2005). Studies conducted by Wongsrichanalai *et al*. (2007) on microscopy recommended it as a standard reference in reading the efficacy of any new diagnostic methodology.

2.2.1.1 *Limitations of Microscopy*

Despite the interesting sensitivity of this technique, it comes under critical scrutiny at instances where malaria transmission is low as compared to PCR. For instance, a study sampled 379 symptomatic individuals of which microscopy detected *P. falciparum* in 25% of the patients as against 37% by nested PCR (Menge *et al*., 2008). This confirms a limit in performance by microscopy during low and unstable malaria transmission, which is a critical factor in Ghana during the dry season. Moreover, studies like one conducted by Johnston *et al*. (2006) observed that microscopy was able to detect 79 positives out of 174 as compared to 99 malaria parasites for PCR, confirming microscopy as less sensitive than PCR. Moreover, from that very study, the specificity of PCR compared to microscopy was also determined to be high for the former, where PCR was able to detect approximately 25% more specific parasites than microscopy. PCR should be the ideal tool of diagnosing malaria, however, the cost for running individual tests as well as increasing dependence on highly skilled operator makes it not the ideal tool.

2.2.2 Rapid Diagnostic Test (RDT)

RDTs are recommended for rapid diagnosis and highly employed especially in less resourced nations (Wongsrichanalai *et al*., 2007), as they give results within maximum of 2 minutes, it requires no skilled operator, easy to do, no sophisticated equipment and no difficulties in 16nalysing or interpreting results, but are available in kits with needed reagents (Peyron *et al*., 1994; Singh *et al*., 1997), which are not expensive. This assay incorporates an antigen-antibody reaction where monoclonal antibodies target parasites antigens such as Histidine-rich proteins (HRP-1, HRP-2 and HRP-3), parasite lactate dehydrogenase (pLDH) and aldose in peripheral blood. This diagnostic tool is much sensitive (>95%) in detecting *P. falciparum* when there is high parasite density, making it suitable for use in clinical diagnosis, during high transmission

season (WHO, 2000). Moreover, due to the short time required and no dependence on skilled operators, RDT becomes ideal especially in remote communities where microscopy is unavailable (WHO 2004).

2.2.2.1 *Limitations of RDTs*

By comparison, RDTs perform malaria diagnosis without parasite quantitation (Murray *et al.*, 2003) against microscopy that offers both parasitological and hematological information although both are suitable for rapid diagnosis. Meanwhile, the performance of RDTs is affected by manufacturing process and environmental conditions (Bell *et al*., 2006; Mboera *et al*., 2006). The recommended temperature range for RDTs is 30-40 ^{o}C, however, tropical temperatures are above 30 ^{o}C and humidity above 70%, impeding their performance as experienced in both field and laboratory diagnosis (Wongsrichanalai *et al*., 2007). RDTs are not effective in diagnosing malaria patients during dry seasons as shown by a study, where out of 90 samples, 2 were identified positive by RDT compared to 42 by microscopy (Azumah *et al*., 2012).

2.2.3 Polymerase Chain Reaction (PCR)

This is a molecular test which is highly sensitive and specific in malaria diagnosis thus very reliable in detecting low or moderate level of parasitemia even to the point of detecting a single parasite per microliter of blood (Johnston *et al*., 2006; Al-Mekhlafi *et al*., 2011). This technique makes use of double stranded parasitic DNA which is initially denatured at temperatures as high as 92°C producing single stranded DNA fragments. PCR also incorporates the use of primers characteristic to the target DNA, being highly specific in annealing to the target sequence leading to the second step. The pairing between the primers and target DNA are

further amplified through a process called extension, making use of a moderate temperature at which the enzyme, DNA polymerase, works best (Dale and Park, 2004). This enzyme is robust and effective at varying temperatures even at high temperatures and requires an environment made up of magnesium ions (Mg2+), and nucleotides (dATP, dTTP, dCTP, dGTP) (Tamarin, 2001).

2.2.3.1 *Limitations of PCR*

PCR is however not considered for routine diagnosis of malaria in tropical regions due to the expensive equipment, long time taken to complete diagnosis, dependency on skilled operator and the cost for a single test. Thus, it is used as a reference diagnostic technique for parasitological confirmation of clinical diagnosis and for research purposes. Moreover, PCR faces the problem of distinguishing viable parasites from non-viable ones due to its unique sensitivity hence not a convincing methodology for the evaluation of the progress of any drug therapy and for clinical diagnosis (Hanscheid, 1999). PCR can also create clinical confusion due to its high sensitivity, for instance, in sub-Saharan Africa where individuals are believed to be carriers due to their immunity, and malaria symptoms evident above 10,000 parasitic counts corresponding to 0.2% parasitemia (Wilkinson *et al*., 1994). On the other hand, PCR has been identified to be effective for accurate malaria diagnosis in geographical areas where parasitemia levels are low (Amexo *et al*., 2004; Hay *et al*., 2004).

Currently, it is recommended that anti-malarials should not be given to febrile patients unless the parasitological presence of malaria has been confirmed by microscopy or rapid diagnostic testing (D'Acremont *et al*., 2009). Meanwhile, this was not the case some years back, where the norm for treating any febrile patient in Africa, was with anti-malaria drug agents (D'Acremont *et al*., 2009). Subsequently, this has reduced the unnecessary administration of

anti-malaria drugs as studies confirm the initial act, presumptive diagnosis, as a contributor to the emergence of drug resistance in malaria parasites (Wernsdorfer, 1994; Payne, 1988). Patients who present with a fever but do not have malaria are more likely to be given an appropriate treatment more rapidly. This revised approach to malaria diagnosis may have a positive impact on patients' perceptions of anti-malarial therapies. Patients will have greater confidence in malaria chemotherapy and the health care system, if it is shown to work quickly and effectively. Accordingly, it is vital that the initial diagnosis is correct to encourage future compliance

2.3 Malaria Treatment Failure and Drug Resistance

Since 2004, the recommended mode for treating malaria by World Health Organization and Ghana Health Service is artemisinin-based combination therapy (ACTs) rather than mono-therapies (MOH, 2007). Historically, a variety of approaches have been used, some of which are ineffective today due to parasite resistance. Chemotherapy is the most effective treatment method and several antimalarial drugs have been recorded to have been used from ancient times to treat the disease. In 1820, the alkaloids cinchonine and quinine were extracted from powdered fever tree (*Cinchona ledgeriana*) bark; prior to this, the dried bark was ground into fine powder and made into a mixture using water which was then drunk to manage the disease (Siegel and Poynter, 1962). Further advancements in malaria treatment were recorded in 1834, where a German physician, Carl Warburg invented an anti-pyretic medicine which contained quinine and a number of other herbs. This treatment method was later referred to as “Warburg Tincture”. The new treatment was eventually considered to be more potent and economical than quinine and therefore was widely used (Poser and Bruyn, 1999).

The aforementioned drugs mark the epoch when natural products were used to treat malaria before being replaced by synthetic mass-produced compounds. William Henry Perkins in 1850 kick-started the concept of synthetic anti-malaria drugs by synthesizing quinine in a commercial practicable process, although unsuccessful, a dye by name 'Perkins Mauve' was produced (Seeman, 2007). This discovery led to important betterments in identification and characterization of potential candidates of chemotherapeutic agents effective against malaria parasites. For instance, methylene blue was noted by Paul Ehrlich to have high affinity for some tissues and also had antimicrobial properties, like preventing the bio-crystallization of haem (Wainwright and Amaral, 2005). In the 1930s Johann "Hans" Andersag and colleagues succeeded in producing Resochin (Dünschede, 1971) as a substitute for quinine which was officially named chloroquine (Loeb, 1946), and was chemically related to quinine in the possession of a quinolone nucleus and the dialkylaminoalkyl amino side chain (Coatney, 1963).

Chloroquine was later found to be an inhibitor of hemozoin production through bio-crystallization and known as one of the best antimicrobials ever developed (Orjih and Fitch, 1993). Chloroquine was known to affect the gametocyte stage of their life cycle; hematin-pigment (hemozoin) is formed as a byproduct of hemoglobin degradation, of malaria parasites. Due to the unique mode of action of chloroquine, it took about 19 years for malaria parasites specifically, *P. falciparum* to develop resistance to the cheap and effective anti-malaria drug (Wellems and Plowe, 2001). The first chloroquine-resistant strains were detected around the Cambodia-Thailand border and in Colombia, in the 1950s (Payne, 1987). With the emergence of chloroquine resistant *Plasmodium* strains, the Chinese government tasked investigators to find the appropriate treatment to eradicate resistant malaria parasites including sensitive ones. Thus, artemisinin was extracted by Tu Youyou, the leader of the team of investigators from the

herb *Artemisia annua* and was presented at the United Nations Scientific meeting in Beijing in the year 1981 (Tu, 1981).

Hereafter, artemisinin became the standard treatment for malaria as it is a sesquiterpene lactone, a peroxide containing group, which is believed to be essential for its anti-malarial activity (RSC, 2006). Derivatives like artesunate and artemether, have been used since 1987 for clinical treatment of drug-resistant and sensitive malaria parasites, especially, in cerebral malaria. These drugs were characterized by their fast action, high efficacy and good tolerance, and their ability to kill asexual forms of *P. berghei* and *P. cynomolgi* including transmission-blocking activity (Chotivanich *et al*., 2006).

Artemisinin-based combination treatments (ACTs) are now widely used to treat uncomplicated *falciparum* malaria, but access to ACTs were initially limited in most malaria-endemic countries and only a minority of patients who needed artemisinin-based combination treatments actually received them (Nosten and White, 2007). Meanwhile, this enigma was made simple after countless studies identified availability and affordability of the effective drug as limiting factors toward malaria eradication and control program. Finally, a global high level subsidy occurred in April 2009, after five years of uncommon debate on the said topic (Laxminarayan and Gelband, 2009). This helped reduce the world mortality and morbidity, which is reflected in a drastic decline in the current worldwide malaria incidence.

Meanwhile, due to the unavailability of health facilities located in remote settings mostly in developing countries (Idowu *et al*., 2006) like Ghana, most inhabitants resolve to the use of herbs in malaria treatment. Herbal remedy is customary in most African communities due to a

host of factors like cost and limited or absence of side effects, as against the itchy and ineffectiveness of anti-malarials like Chloroquine. A study conducted in Lagos identified above 50% of the 1,593 respondents, attesting to the almost complete effectiveness regarding herbal remedy of malaria infection (Idowu *et al*., 2006). As compared to a study conducted in 70 children in Ghana with cerebral malaria which encountered significant frequencies of treatment failure in patients who received chloroquine (Goka *et al*., 2003). This however raises concerns to communities that still use herbal remedies spiked with chloroquine.

The treatment of malaria has been faced with several challenges which continually accounts for the increasing percentages of treatment failures, over and over again. These hindrances to the treatment of malaria threaten the overall objective of completely eradicating the disease. Some of these challenges include misdiagnosis of malaria (diagnostic errors), non-compliance of patients, self-medication of substandard or fake anti-malarial drugs and the development of drug resistance by the malaria parasite. A major challenge to achieving an effective treatment plan for malaria is patients' attitude to chemotherapy, critically non-compliance, which has been strongly associated with the development and endemicity of drug resistant parasites (Fernandez *et al*., 2011; Nsungwa-Sabiiti *et al*., 2004). Most patients stop the intake of antimalarial drugs after the symptoms of the infection begins to abate, although the parasites might not be completely removed. Eventually, as confirmed by a study done by Souares *et al*. (2009), non-compliance mostly emanates from the lack of trust, on the part of patients in the health care system provided in resource poor communities.

In as much as self-medication is known to be customary in Ghana, it is the first line of treating any infection especially in the rural communities with no or limited access to health care

facilities. The use of over the counter antimalarial drugs has been found to contribute to the loss of faith in orthodox drugs (Ukaga *et al*., 2006), like Chloroquine. One can also use the upsurge of treatment failures to explain the lack of trust in the health care system of impoverished countries. Treatment failure occurs when a particular anti-malaria drug is unable to kill malaria parasites, which is very common at the moment considering Chloroquine which lost the killing power some years ago, after dominating as the recommended drug for treating uncomplicated malaria.

Counterfeiting of drugs has been found as a contributor to the high treatment failures of chloroquine as well as the emergence of resistance. The subject of fake drugs has been ignored or at least largely underestimated. It has now clearly emerged as one of the most crucial public health problems mainly for malaria treatment in developing countries (Aldhous, 2005). Poor quality drugs have been a persistent problem starting in the 1600s and 1800s with fake cinchona bark and quinine, respectively (Bate *et al*., 2008). Until now artemisinin is mainly produced in Asia, with unfortunately a very large proportion of fake products (Newton *et al.*, 2006) due to the shortage of the natural plant material *(Artemisia annua*) from which artemisinin is extracted. The situation is even more dramatic in sub-Saharan Africa where the burden of malaria is the greatest. A large proportion of these fake anti-malarials are imported from Asia (Ambroise-Thomas, 2012).

2.4 Chloroquine Resistance

There has been intensive laboratory and field studies to elucidate the major mechanism behind drug resistance in malaria parasites, *P. falciparum*, which they finally identified the multidrug resistance gene (*pfmdr1*) (Su *et al*., 1997) and the chloroquine resistance transporter gene

(*pfcrt*) (Fidock *et al*., 2000). The first chloroquine-resistant strains were detected around the Cambodia-Thailand border and in Colombia, in the 1950s (Wongsrichanalai *et al*., 2002). These resistant strains spread rapidly, resulting in a large increase in mortality from malaria particularly in Africa during the 1990s (Bate *et al*., 2008).

The development and spread of resistant strains to certain anti-malarial agents, specifically chloroquine, has interfered with malaria eradication programs and has probably contributed to the resurgence of infection and the increase in malaria-related deaths in recent years (WHO, 2008). Resistance to almost all commonly used anti-malarial, notably chloroquine and sulphadoxine-pyrimethamine, (but also amodiaquine, mefloquine, and quinine), has been observed in the most lethal of the parasite species, *P. falciparum* (WHO, 2008). The problem of resistance exists in greater parts of Africa and Southeast Asia (White, 2004). For example, treatment failure rates of about 70-80% have been reported for chloroquine (White, 2004), which was formerly the cheapest and most widely used anti-malarial drug. Currently, there is increasing drug resistance to amodiaquine in large parts of East Africa, potentially rendering the combination of artesunate and amodiaquine less effective (WHO, 2008).

The half-life of an anti-malarial is believed to be an important factor in the development and spread of resistance (Hastings *et al*., 2002). It has been suggested that compounds with a longer half-life have a greater propensity to become ineffective due to parasite resistance. For a drug that has a half-life of weeks or months (e.g. mefloquine, piperaquine or chloroquine), there is a slow elimination from the host's blood which eventually enables parasites to be exposed to residual drug concentrations. Malaria parasites that persist during that time frame can easily develop resistance as they are exposed to partially effective drug concentrations (White, 2004).

However, it has also been proposed that a longer half-life offers protection against re-infection for a longer period of time - this effect is known as "post-treatment prophylaxis". It represents the period of time after an anti-malarial treatment dose, during which re-infection is suppressed (White, 2004). Consequently, infected patients are faced with greater risk of unsuccessful treatment as more parasites are exposed to sub-therapeutic drug levels.

Suspicion of the presence of chloroquine-resistant strains of *P. falciparum* in Ghana was documented as far back as 1965; however, these reports were not confirmed. In 1984, Ofori-Adjei *et al*., reported on the *in vivo* and *in vitro* sensitivity patterns of *P. falciparum* to chloroquine in three communities in Ghana. In 1986, Neequaye reported an *in vivo* resistance to chloroquine in a sickle cell disease patient who had also been on chloroquine prophylaxis. The patient was given chloroquine in a dose greater than the recommended with no significant reduction in parasite density 48 hours after initiation of the therapy. One important finding in the study was that as one moved away from the urban to the rural area, the percentage of resistance to chloroquine decreased. The explanation offered by the authors was that drug pressure was more pronounced in urban areas than in the remote regions whereas drug pressure has been noted as a contributor in the selection of resistant strains. Ofori-Adjei *et al.* (1984) called attention to the indiscriminate use of chloroquine in the urban populations of Ghana and elsewhere in the sub region of West Africa (Ofori-Adjei *et al*., 1984).

2.4.1 Mechanism of Chloroquine Resistance

In the erythrocyte stage of *P. falciparum*, a lysosomal isolated acidic compartment known as the digestive vacuole (DV) which serves as the compartment in which the parasite deposits haemoglobin from the host red blood cells. Haemoglobin is then disintegrated into its

component peptides and haeme, inside the compartment and is incorporated into the inert and harmless crystalline polymer hemozoin (Slater *et al*., 1991). It is through this compartment that most drugs, chloroquine for example, target to disrupt the life cycle of the parasite.

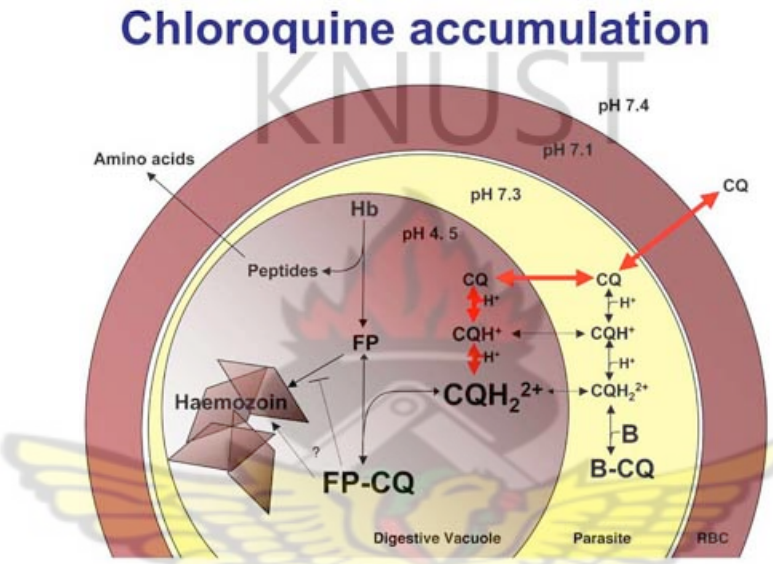

Figure 2.3: Chloroquine Accumulation in Resistant *P. falciparum*

The image above describes the sequestering mode of chloroquine inside the digestive vacuole (DV) of malaria parasites. Chloroquine accumulates inside the parasites cytoplasm rather than its digestive vacuole in resistant strains whereas otherwise in sensitive ones (Hladky, 2008).

In the host system, chloroquine can exist in three forms at physiological pH, for example un-protonated: which is the uncharged chloroquine form and is the only membrane permeable form of the molecule and it freely diffuses into the erythrocyte up to the digestive vacuole (DV). Parasites sensitive to chloroquine mostly referred to as "Chloroquine sensitive parasites"

(CQS) accumulate much more chloroquine in the DV than chloroquine resistant strains (CQR) (Saliba *et al*., 1998). The reduced chloroquine accumulation observed in the parasite vacuole of resistant strains (Saliba *et al*., 1998) has been associated with point mutations in the gene encoding for a transporter protein called the *P. falciparum* chloroquine resistance transporter (PFCRT) protein. The PFCRT protein is localized in the digestive vacuole membrane and contains 10 predicted membrane-spanning domains (Cooper *et al*., 2007).

CQR phenotype isolates have also been found to carry the PFCRT critical charge-loss mutation lysine to threonine (K76T) or, in two single cases, K76N or K76I (Huaman *et al*., 2004). Chloroquine sensitivity in resistant parasites has been recorded to be restored by another mutation, serine to arginine (S163R), (Johnson *et al*., 2004). The K76T amino acid mutation might allow the interaction of PFCRT with the positively charged chloroquine (CQ+ or CQ++) and allow its exit from the vacuole, with the net result of decreasing the chloroquine concentration within the DV (Martin and Kirk, 2004). The single amino acid change S163R caused by reintroducing a positive charge, is thought to block the leak of charged chloroquine from the DV, thus restoring chloroquine sensitivity (Lehane and Kirk, 2008). Both wild-type and resistant forms of the PFCRT protein have demonstrated that chloroquine resistance is due to direct transport of a protonated form of chloroquine out of the parasite's DV via the K76T PFCRT mutant (Pagola *et al*., 2000). However several experiments have failed to answer the question on whether PFCRT protein is a channel or a carrier.

Chloroquine accumulation is an energy dependent process in both CQR and CQS strains (Sanchez *et al*., 2003) was demonstrated by researchers to determine chloroquine uptake in the presence and absence of glucose. They found that, after glucose addition, the time courses of

chloroquine uptake were significantly different in CQS and CQR: chloroquine accumulated to an increased extent in the CQS strain, but decreased in the CQR strain. After similar experiments with other antimalarial drugs it was concluded that some energy-dependent mechanisms lead to the loss of chloroquine from CQR cells and to its accumulation in CQS parasites (Sanchez *et al*., 2003). However, Bray *et al*. in 2006 measured cellular accumulation ratio of chloroquine in different experiments and concluded that chloroqusine movement through PFCRT protein is not an active process. Results of several other experiments have failed to arrive at a consensus as to the nature of the *pfcrt* gene.

2.4.2 Testing for Chloroquine Sensitivity

To determine the sensitivity of *P. falciparum* to chloroquine within any population, a molecular based analysis of parasitic DNA or *in vitro* drug test is an appropriate method (Sanchez *et al*., 2003). Furthermore, to determine whether the resistant parasite carries the K76T mutation, an amplification of parasitic DNA coupled with restriction digest is the easiest method. Restriction digestion of DNA is done using a restriction enzyme, for example APO-1 or XAP-1. These are endonucleases that cleave at the beginning of the nucleotide sequence AAATTT in the wild type strain of the parasite. The mutant strain (resistant strain) bearing the K76T mutation has this site disrupted and hence cannot be digested by the restriction enzyme. This was shown in studies done by Abruquah *et al*. (2010) and Azumah *et al*. (2012) where the restriction enzymes did not digest a DNA of 145 base pair (bp) length to produce two fragments of length 114bp and 31bp for the wild type, whereas single fragments of 145bp length were obtained for mutant strains (meaning they were not cleaved by the enzyme). This outcome was also observed in a study done by May and Meyer (2003) where the mutant strain DNA remained un-fragmented after a restriction digest.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area and Collection of Blood Samples

Malaria parasites, specifically *Plasmodium falciparum*, were randomly sampled from patients from four (4) different communities in Ashanti region, with two representing urban centres, whereas the other two represented rural centres. The two urban centres, Komfo Anokye Teaching Hospital (KATH) and the Kwame Nkrumah University of Science and Technology (KNUST) Hospital were chosen due to their close proximity to well stocked chemical shops. The results from these centres were compared to the two rural centres, Nkwantakese and Pampatea, two malaria endemic communities which are situated about 25km north of the aforementioned urban centres. Pampatea is relatively more rural than Nkwantakese. The primary occupation of the rural inhabitants is farming. Unlike the urban centres, these rural communities did not have easy access to well stocked chemical shops. After securing permission from the Afigya Kwabre District Health Directorate, sampling was done from December, 2012 to January, 2013 and stored at the Department of Clinical Microbiology at the School of Medical Sciences until analysis.

Thick blood smears and spots were used to ascertain the presence of resistant and sensitive malaria parasites from sampling locations using microscopy as the gold standard methodology. Blood samples from patients visiting the urban centres (KATH & KNUST-Hospital), for routine malaria diagnosis and treatments, were used for this study. Microscopic diagnosis used by the technicians was documented and matched blood spots on filter papers were taken. The blood spots were prepared by blotting about two to three drops of blood on a filter paper, air

dried and stored in mini tablet bags at room temperature for DNA extraction. A total of 205 blood spots were collected from KATH (100) and KNUST-Hospital (105).

Additional 203 blood spots and smears were sampled from Nkwantakese (123) and Pampatea (80), at their community clinics giving a total sample size of 408. Participants were patients visiting the community clinics for routine malaria intervention which was basically by presumptive diagnosis. At these health units, blood samples obtained by thumb prick were placed on fresh slides, air dried and transported to the haematology laboratory at KATH for confirmation by thick smear analysis. Moreover, their corresponding blood spots were taken and stored as described earlier. Of the 408 thick smear readings and blood spots sampled for the study, 5 samples from KNUST-Hospital were excluded from the final analysis due to loss of quality with the blood spots.

3.2 Malaria Thick Smear Analysis

A drop of blood was spotted and smeared onto a clean labelled glass slide after which the slides were air-dried and stained using 5% of Giemsa stain which is prepared using alcohol as the buffer. Giemsa is a dye which stains malaria parasites (purple) differently from that of red blood cells (blue). Giemsa stained thick smears are diagnosed for malaria parasites using a light microscope, under the 100x oil immersion objective and rated using the 'plus system' of counting. According to this system, a plus sign +, is used to denote the presence of malaria parasites, in an increasing manner depending on the number of parasites seen in a set field. A single +, also represented as 1+, implies very low number of parasites present in the sample, usually between 1-10 parasites. The highest parasite density is labelled ++++ (4+), whereas 0 or negative (-) is used to represent the absence of malaria parasites.

3.3 Nested-PCR for Malaria Diagnosis

The nested Polymerase chain reaction (Nested PCR) was used to determine the presence or absence of malaria parasites, *P. falciparum*. This is a modification of polymerase chain reaction (PCR-process of amplifying DNA samples) in order to prevent or reduce non-specific binding in the final products due to the amplification of unexpected primer binding sites. Nested PCR was used as a confirmatory tool for thick smear analysis. Furthermore, the products of the nested-PCR were used to ascertain the abundance of a mutation at the 76th position of the *P. falciparum* chloroquine resistance transporter gene (*pfcrt*). To achieve the above objectives, genomic DNA was extracted, amplified using two sets of primers, digested with a restriction enzyme, and finally analysed using an agarose gel electrophoresis.

A punch was used to cut a portion of the blood spots from the filter papers into well labelled microfuge tubes properly arranged in a rack. Eighty microliters (80 µl) of Tris-EDTA (TE) buffer was added to the punches making sure they were completely immersed. Samples were then incubated at 50 ºC for 15 minutes, and moved to a 97 ºC heat block, for a final incubation of 15 minutes. The samples were then centrifuged briefly and ten microliters (10 µl) of supernatant was transferred into a well-labelled microfuge tube for storage at -20 ºC until PCR was done.

A reaction volume of 25 µl made up of 5 µl DNA template (sample), 15 µl primer mix (deionized water and primers labelled P) and 5µl lyophilized master mix (Taq polymerase, dNTPs, MgCl2 and buffer) was used. The pair of primers used for the primary PCR was CCG TTA ATA ATA AAT ACA CGC AG (23) and CGG ATG TTA CAA AAC TAT AGT TAC

C (25), representing forward and reverse primers respectively. For the negative control, 5µl of deionized or nuclease free water was used in place of the sample. The primary run made use of a set of primers that initially binds to the 2nd exon of the *Plasmodium falciparum* Chloroquine transporter *(pfcrt*) gene to produce amplicons of length 537 bp. For this run, templates were initially denatured at 94 ºC for 3 minutes before annealing with the primers at 56 ºC for 30 seconds. It took about a minute for extension of the DNA amplicons to take place at 60°C , with the final extension lasting 3 minutes, [94 °C, 3 min; 94 °C, 30 sec; 56 °C, 30 sec; 60 °C, 1 min; 72 °C, 3 min final extension]. The entire run went through 30 cycles which lasted about an hour and 45 minutes.

Before undergoing the second run, gel documentation was performed to confirm the effectiveness of the entire setup. A 5 µl amplicons from the primary run was pipetted into new sets of PCR reaction tubes, already having the primer mix. The upstream and downstream primers for the nested-PCR were T GTG CTC ATG T GT TTA AAC TT and ACA AAA TTG G TA ACT ATA GT TTT G respectively. Amplicons from the primary run were templates for the nested run, which were then melted at 95 ºC for 5 minutes before annealing to the nested primers at 48 ºC for 30 seconds. Initial and final extensions of nested amplicons took place at 65 ºC under 30 seconds and 3 minutes respectively, [95 °C, 5 min; 92 °C, 30 sec; 48 °C, 30 sec; 65 °C, 30 sec (25 cycles); 72 °C, 3 min final extension]. The nested PCR underwent 25 cycles culminating in products (amplicons) of 145 bp in length. Care was taken during loading and offloading of the thermal cycler to prevent any form of crossover.

After the nested run, amplicons were analyzed using agarose gel documentation. A 2 % agarose gel was prepared from 1.0 g of agarose dissolved in 50 mls of Tris-acetate EDTA (TAE) buffer

that contained 5 µl of a fluorescent dye (Gel red). Using a DNA-ladder of 50bp, 10 µl of amplicon was loaded into the wells, one-well to one-sample. No loading dye was used as the PCR reaction contained its own dye (manufactured). The gel electrophoresis was used to resolve bands of length 145bp which threw much light on the presence or absence of malaria parasites in the respective samples. This methodology was used as a reference diagnostic tool to the microscopic readings of blood smears.

3.3.1 Restriction Digest of Nested PCR Products

After the first gel documentation, samples (amplicons) identified positive for malaria parasites by nested-PCR were digested using XAP 1 restriction enzyme. One microliter (1µl) of restriction enzyme was pipetted into a digest tube containing 3 µl of nuclease free water and five microliters (5µl) of positive samples were then added and digest tubes were placed in a heat block at 90 °C for 15 minutes. The restriction enzyme recognized sequences AAG or AAA that code for lysine. Amplicons bearing sequences different from that of lysine at the 76th position located in the 145bp product were not digested. The final step was a second gel documentation of digested bands to distinguish between resistant and sensitive strains. A resistant band had length of about 145bp since there was no digestion whereas a sensitive sample produced 2 bands of length 114 and 31bp. This was because the sensitive strains bore the sequence AAA or AAG at the 76th position.

3.3.2 Data Storage and Analysis

Microscopic readings were documented and analysed using Microsoft excel spread sheet 2013 version. Routine microscopic diagnosis from the two labs (KATH & KNUST) was compared to those of nested-PCR to estimate the level of concordance. Specific errors of microscopy were determined using PCR as reference, that is, the positive and negative predictive values, which are basically the ability of the diagnostic tool to accurately detect the samples that are truly positive and negative respectively. Positive predictive value (PPV) was estimated using the total number of true positives divided by the sum of true positives and false positives, and vice versa for the negative predictive value (NPV). Furthermore, the specificity (proportion of actual negatives confirmed) and sensitivity (proportion of actual positives which were accurately identified) of microscopy were estimated. To determine specificity of microscopy, number of true negative samples obtained by microscopy was divided by actual negatives obtained by PCR whereas sensitivity was true positives obtained by microscopy divided by true positives by PCR. Specificity and sensitivity of microscopy were used to determine type-I and type-II errors using respective diagnosis from nested-PCR as reference (Peck and Jay, 2011).

Percentage sensitivity and resistance of malaria parasites were estimated from positive samples by nested-PCR and compared between all four locations. Correlation between the availability of chemical shops and the percentage resistance and sensitivity were finally estimated.

CHAPTER FOUR

4.0 RESULTS

4.1 Malaria parasites detected by PCR had a higher frequency than by thick smear

Out of the 403 blood samples collected, 27 (7%) and 376 (93%) smears were observed by the microscopic technicians as positive and negative respectively, as compared to 39 (10%) positives and 364 (91%) negatives by PCR (Table 4-1). Out of the 27 positives by thick smear, 13 were consistent with PCR whereas 14 were rather negative by PCR (Table 4-1). On the other hand, 26 out of the 376 negative samples by thick smear were found positive by PCR with the remaining 350 confirmed negative (Table 4-1).

Table 4.1 Consistency of thick smear diagnosis relative to PCR

Smear (403)	*PCR+(39)*	*PCR-(364)*
Positive **(27)**	13	14
Negative **(376)**	26	350

4.2 More errors were made by thick smear when detecting negative samples

Individual microscopic ratings, from 0 to 4, for thick smears sampled from the respective locations (KATH, KNUST-Hospital, Pampatea and Nkwantakese), were compared to that of nested-PCR. Basically, out of the 27 positives by microscopy, 20 thick smears were rated +1 followed by 5 and 2 thick smears rated +2 and +3 parasitemia levels respectively (Table 4-2). None of the thick smears were diagnosed at +4 parasitemia. Out of the 100 smears sampled from KATH, 98 thick smears were diagnosed negative with 2 positives at +2 parasitemia levels

by microscopy (Table 4-2). On the other hand, PCR identified 91 negatives and 9 positives, of which just 1 PCR positive was in agreement with that of microscopy (Table 4-2).

The highest number of positives was observed for the 100 thick smears sampled from KNUST-Hospital, 20 positives as against 80 negatives, with 17 of the positives rated at 1+, 2 at 2+ and 1 at 3+ (Table 4-2). However, PCR identified 14 positives for KNUST-Hospital out of which 9 were validated by PCR as positive. Considering the rural centres, out of 80 smears sampled from Pampatea, 77 were rated negative with 3 positives each rated at 1+, 2+ and 3+ (Table 4-2). The highest number of negatives by thick smear, for any particular location, was observed for Nkwantakese (121 out of 123) as against 110 negatives by PCR. And, 2 positives all rated at 1+ were observed by thick smear with 13 positives identified by PCR respectively (Table 4-2). Of the 2 positives by thick smear, only one was validated by PCR (Table 4-2).

The three (3) thick smears rated positive by microscopy above 1+ parasitemia (2+ & 3+) all correlated with that of nested-PCR but only 6 out the 17 positives at 1+ agreed. The least number of positives according to microscopy was observed for samples from KATH and Nkwantakese with PCR identifying samples from Pampatea to have the least positives, 3 (Table 4-2). In comparing microscopy to PCR, 26 (7%) thick smears were falsely diagnosed negative whereas 14 (4%) samples were rated positive instead of negative (Table 4-2). Out of the 27 positive samples by thick smear, no sample was diagnosed at +4 parasitemia whereas 2 samples were rated +3 with 5 at +2 and the remaining 20 samples at +1 (Table 4-2). With reference to PCR, 7 out of the 20 at +1 parasitemia were confirmed positive and 4 out of the 5 at +2 parasitemia were consistent (Table 4-2). The level of concordance regarding smears rated at +3 parasitemia was 100% (2 out of 2) (Table 4-2). Forty (40) smears were falsely diagnosed

by microscopy according to PCR out of which 26 and 14 smears were falsely diagnosed positive and negative respectively.

Table 4.2 Samples diagnosed by thick smear and those confirmed by nested PCR

Locations-Total	Thick Smear **[*PCR-validated*]**					
	0	**1**	**2**	**3**	**4**	**FD***
KATH-100	98 **[*91*]**	0	2**[*1*]**	0	0	*8**
KNUST-100	80 **[*75*]**	17**[*6*]**	2**[*2*]**	1**[*1*]**	0	*16**
PAM-80	77 **[*75*]**	1 **[*0*]**	1**[*1*]**	1**[*1*]**	0	*3**
NKWAN-123	121 **[*109*]**	2 **[*1*]**	0	0	0	*13**
Total	376 **[*350*]**	20**[*7*]**	5**[*4*]**	2**[*2*]**	0	***40****

KATH represents Komfo Anokye Teaching Hospital, **KNUST** denotes Kwame Nkrumah University of Science and Technology-Hospital (KNUST-Hospital), **PAM** for Pampatea and **NKWAN** for Nkwantakese. Also, + and – represent positive and negative diagnosis. Figures in parenthesis denote samples confirmed by PCR. False diagnoses were labelled with **FD***.

4.3 Microscopic errors using nested-PCR as reference standard

Out of the 403 thick smears sampled from the aforesaid locations, a negative predictive value (NPV) of 0.93 (93%) was recorded for microscopy as against 0.481 (48%) positive predictive value (PPV) (Table 4-3). Furthermore, the sensitivity and specificity of microscopy were assessed using nested-PCR as a reference standard. A sensitivity of 0.333 (33%) was ascertained using the true positives by microscopy and actual positive by nested-PCR (Table 4-3). A high specificity value of 0.961 (96%) was estimated for microscopy as compared to the standard [PCR] (Table 4-3). These outcomes of sensitivity and specificity facilitate the determination of the extent of errors made by microscopy. Thus, using the specificity value of

0.961, eventually, a low type-I error of 4% (0.038) was estimated whereas a high type-II error of 67% (0.667) was determined (Table 4-3).

Table 4.3 Microscopic errors using nested-PCR as reference standard

Microscopy	Predictive Value		Diagnostic Errors			
	PPV	NPV	Sensitivity	Specificity	Type I	Type II
Positive (27)	0.481	-	0.333	-	0.039	-
Negative (376)	-	0.93	-	0.961	-	0.667

PPV and NPV represent Positive and Negative Predictive Values

4.4 Parasites sampled from the rural settings were not less resistant than those from the urban setting

Out of the 39 positive samples by PCR, 23 (59%) were from the respective urban centres (KATH & KNUST-Hospital) and 16 (41%) for the rural sites (Pampatea and Nkwantakese) (Table 4-4). All 9 positive samples from KATH were all found to be resistant whereas out of the 14 positives from KNUST-Hospital, only 3 samples contained chloroquine sensitive strain after the restriction digest with Xap-1 as against 11 resistant strains (Table 4-4). The other location to follow in this regard was Nkwantakese, with 12 (92%) out 13 positives being resistant strains (Table 4.4). However, the samples from Pampatea were found to be sensitive (Table 4-4).

Altogether, 4 (10%) out of the 39 positive samples had sensitive parasites of which 3 came from the urban centre (KNUST-Hospital) and 1 from the Nkwantakese (Table 4-4). Conversely, 35 (90%) out of the 39 positives contained resistant parasites, of which 20 came

from KATH and KNUST-Hospital compared to 15 from Nkwantakese and Pampatea (Table 4-4).

Table 4.4 Status of *Plasmodium falciparum* to Chloroquine treatment

Location	PCR-positive		Chloroquine Status	
			Sensitive	**Resistant**
KATH	9	***23***	0	9
KNUST	14		3	11
PAM	3	***16***	0	3
NKWAN	13		1	12
		Total count:	**4**	**35**

KATH represents Komfo Anokye Teaching Hospital, **KNUST** denotes Kwame Nkrumah University of Science and Technology-Hospital (KNUST-Hospital), **PAM** for Pampatea and **NKWAN** for Nkwantakese. Also, + and – represent positive and negative diagnosis.

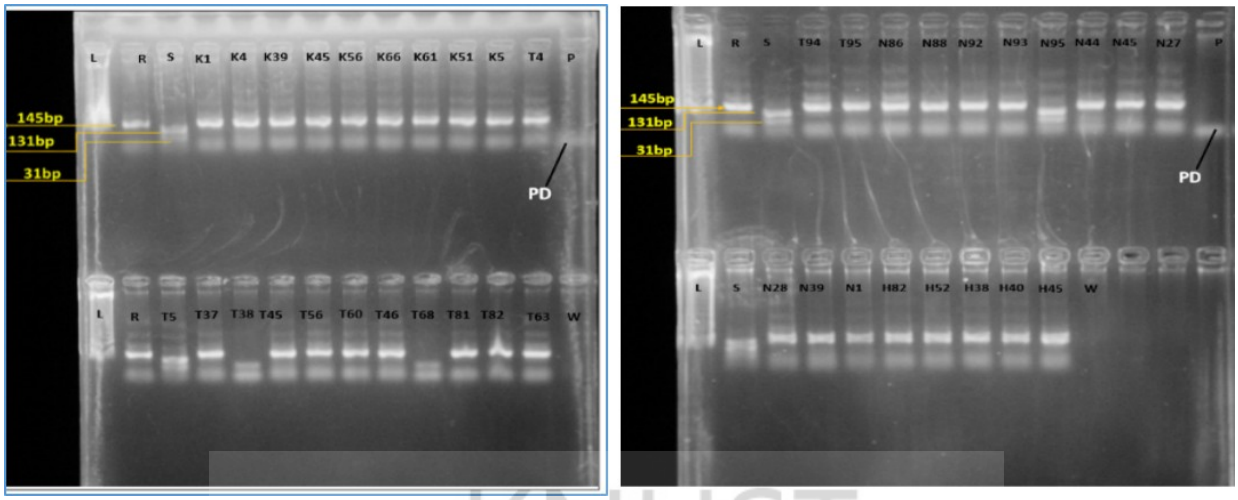

Figure 4.1 Gel scans of products of positive amplicons after restriction digest with Xap-1

The first three lanes of the gels A & B shown above contained the ladder (L), and two positive controls labelled R&S for resistant and sensitive strains respectively (Upper lanes). The lower set of bands bears the ladder (L) and resistant control (A) and sensitive control (B). According to gel A, 21 positive samples were digested after being amplified using nested-PCR. It can be seen that out of the 21 samples, only 3 (samples T5, T38 & T68) were found in the same position as the sensitive (S) control and the remaining 18 were in line with the resistant control (R). On the other hand, on gel B, out of 18 positive samples digested with Xap-1 only one (N95) was positioned in the same lane as the sensitive control (S) with the remaining 17 similar to the resistant control (R). Those in line with the resistant control were in effect not digested by the Xap-1 enzyme. Although the actual size of the bands could not be anticipated by just looking at the gel as the ladder did not properly resolve, the resistant band is at 145bp and the digested ones are 114 and 31bp.

CHAPTER FIVE

5.0 DISCUSSION

This study hypothesized that with a very high chloroquine resistant parasite population in the urban centres of Ghana, the reverse was expected moving further to a more rural setting, that is, the impact of distance on the status of malaria parasites toward chloroquine treatment. Tthe second goal was to compare the two methodologies, thick smear diagnosis under usual working conditions and Polymerase chain reaction (PCR), during a low transmission season.

From the study, out of 403 samples diagnosed for the presence of *P. falciparum*, 39 samples were confirmed positive by PCR of which 23 (58.9%) came from the central and peri-urban communities (KNUST-Hospital and KATH) and the remaining 16 (41.1%) from the rural setting (Pampatea and Nkwantakese). In ascertaining the abundance of chloroquine resistant parasites in all four locations, the 39 positive samples were further digested using the restriction enzyme XAP-1, which produced 35 (89.7%) positive samples bearing the K76T mutation with the remaining 4 (10.3%) having the wild type genome. Overall, high percentage resistance was identified, which is consistent with previous findings by Abruquah and colleagues in 2010, who identified approximately 88% resistance, 124 out of 140 samples in the year 2012 and a bit below 97% resistance (38 out of 39) observed by Azumah *et al*., (2012). This current finding continues to paint the clear picture that approximately 90% of any malaria population, sampled in this country stands the chance of bearing the K76T mutation, which is problematic for malaria treatment, management and eradication in Ghana. This further implies that no improvement has been observed in resistant parasites populations in Ghana after Abruquah's 88% resistance as far back as 2010. The current prevalence (90%) of chloroquine resistant parasites continues to exclude this country from the convenient landmarks achieved by the likes

of Tanzania and Kenya regarding the return of chloroquine sensitivity (Frosch *et al*., 2011). These African countries did follow Malawi and had similar outcomes but not so for Ghana, thus far. However, no concluding facts can be completely drawn from these findings as few positives were obtained out of the 403 samples used in the study.

Moreover, out of the 35 positives confirmed to have the mutant sequence, 20 came from the central and peri-urban centres with the remaining 15 from the rural centres. Meanwhile accessing the percentage abundance of resistant strains from these locations, parasites sampled from the rural communities (Pampatea and Nkwantakese) were more resistant, 15 out of 16 that is 94% (Table 4.4), compared to an incidence of 87% (20 out of 23) for the urban and peri-urban centres (KATH and KNUST-Hospital). This was the exact opposite of the expected outcome as distance did not play a critical role in chloroquine sensitivity in *P. falciparum* population, although there were far too few malaria positives (39 out of 403), for one to observe any statistical significance. However, such outcomes may be associated with the fact that most rural inhabitants still have access to chloroquine, if not limited, and also the continual use of chloroquine containing herbal extracts (Idowu *et al*., 2006). The latter is more likely to be a major contributor, as the use of Chloroquine-related phytochemicals to treat uncomplicated malaria in the rural communities is habitual. However, the former has been confirmed by a study conducted at the southern part of Ghana by Asare and colleagues (2013), according to the study, approximately 17% of participants tested positive for the presence of chloroquine metabolites in their urine. Furthermore, 10 out of the 69 chemical shops sampled were found to have stocks of chloroquine and confirmed routine patronization by inhabitants.

The persistent rate of chloroquine resistant *P. falciparum* population in Ghana, though high, suggests that sensitivity of parasites to chloroquine is not likely to return any time soon. The concept of most Ghanaians still using chloroquine to treat malaria infection is still alarming, most especially, in communities having limited insight on the drug's current treatment failure in clearing resistant parasites, as noted in 94% (15 out of 16) for the rural communities (Asare *et al*., 2013). This trend indicates that it is imperative for the Ghana Health Service (GHS) to embark on educating rural inhabitants on the need to switch from the use of the ineffective chloroquine and stick to the current recommendation of ACTs. In addition, it can be inferred that chloroquine sensitivity is far from being attained in Ghana, 10 years after chloroquine was removed as the first line drug for malaria treatment. Thus, this country cannot join the likes of fellow African countries, Kenya, Tanzania and Malawi, who have successfully achieved a reversion in chloroquine sensitivity, 10 years after restricting the use of the drug in their countries (Frosch *et al*., 2011).

With microscopy, the 'plus scale' count was used to rate thick blood smears from 0 to +4. Thick smears were scored 0 if there were no parasites observed under the microscope, of which 376 out of the 403 thick smears corresponding to 93.3% were found to be negative. On accuracy of diagnosis, thick smear identified approximately 7% (27) of the 403 slides as positive, though few, not all that alarming as sampling was done during the dry season, and at a time where there has been reports of worldwide decline in annual malaria incidence (WHO, 2013). Comparing to PCR which is known to offer a more higher sensitivity than smear based diagnosis, (that is 0.4 parasites/ µl of blood as against 1-10 parasites/ µl of blood), out of the 403 samples, 39 positives were identified by PCR. Thus, a malaria incidence of 10% was observed for this study which is a bit below the current worldwide incidence of 16% (Guillebaud *et al*., 2013).

Microscopy was able to detect 27 positives with a sensitivity of 1-10 µl (Bain *et al*., 1997), however, with a sensitivity of 0.4 parasites/ µl for PCR (Johnston *et al*., 2006; Al-Mekhlafi *et al*., 2011) PCR should have identified 68 (16.8%) positives. Meanwhile, 12 more positives were detected by PCR (39) compared to that of microscopy (27). Interestingly, this was not consistent with a study in Bangladesh where 72 positives were detected by microscopy compared to 65 by PCR (Fuehrer *et al*., 2011). However, similar results were observed in a study conducted in Brazil where out of 369 samples, 165 (45%) positives by PCR was observed as against 125 (34%) by microscopy (Scopel *et al*., 2004), with a difference of 40 (11%) positives. A possible explanation could be the quality of the PCR setup including optimization of respective PCR profiles. However, this idea can be challenged on the back of time of sampling, in the dry season, and the fact that there is promising data confirming worldwide decline in malaria incidence as of 2013 (WHO, 2013).

Assessing how accurate microscopy was in diagnosing for malaria parasites, it was observed that out of the 27 positives by smear only 13 were confirmed by PCR with the remaining 14 identified as negative (false positive), rather (Table 4.1). Further microscopic tool for malaria diagnosis, according to this study, was deficient in discriminating between negative (0) and +1 parasitemia, which is most critical. Thirteen (13) samples were misdiagnosed as positive at +1 parasitemia and 26 negative by microscopy were found positive by PCR (Table 4.1). Thus, implying that microscopic errors were profound when distinguishing between true negatives (0) and very low parasitemia (+1); which were not far from a previous study that confirm microscopy as not the ideal tool for malaria diagnosis during low transmission (Coleman *et al*., 2006). Microscopy on the other hand was found to be more accurate in diagnosing samples

with high parasite load using PCR as a reference; with 6 out of 7 samples identified positive at higher parasitemia of +2 and +3. Although few positives were obtained for the two methodologies, factors like artefacts and poor smear preparation can account for the errors encountered by microscopy, as confirmed in a study by Wongsrichanalai *et al*. (2007). Storage quality of blood spots and inadequate optimization of the PCR profile can account for the low positives experienced in PCR. However, another point worth noting is the incomparable sensitivity of PCR as it detects parasitic DNA but not the parasite (Najafabadi *et al*., 2014), meaning the 7% (26 out of 376) thick smears identified positive by PCR but negative by microscopy could just be parasitic DNA but not necessarily the parasite. Therefore, the outcome by PCR might not be reliable after all as there can be parasitic DNA as a result of the action of host immune system

Overall, no concrete stance was achieved from this study concerning the exact statistical significance of the rate of resistant *P. falciparum* population, thus, way above 50 percent resistivity was observed. However, the idea of malaria infection and transmission reducing in this country is more profound as this study agrees with previous findings, not forgetting the current worldwide malaria incidence. Finally, according to this study microscopy was somewhat unreliable in distinguishing between the uninfected and those harbouring low parasite counts, during the dry season, which is the critical aspect of malaria diagnosis.

CHAPTER SIX

6.0 CONCLUSIONS

Microscopy as the gold standard is quite efficient but needs expertise to be able to differentiate between samples that are truly negative and those with very low parasite counts. The Polymerase Chain Reaction (PCR) method, even though it has a high level of sensitivity, may not be appropriate for routine malaria diagnosis because of the time, equipment and expertise needed.

6.1 RECOMMENDATIONS

As shown by this study, after 10 years of changing chloroquine as the first line treatment and adopting the Artemisinin Combination Therapy (ACT), there is persistent alarming rate of resistant parasites in Ghana in both rural and urban settings. Eventually, many improvements like drawing concrete statistical significance will be possible with further works aimed at making PCR less operator dependent and cheaper.

CHAPTER SEVEN

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