Anti-malarial drug resistance and associated genetic polymorphism of *Plasmodium falciparum* in members of Armed Forces of Bangladesh working in endemic areas at home and abroad

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Anti-malarial drug resistance and associated genetic polymorphism of *Plasmodium falciparum* in members of Armed Forces of Bangladesh working in endemic areas at home and abroad

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Certificate

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Dedicated to

My beloved parents

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Abbreviations

AA	Artesunate-amodiaquine
ACT	Artemisinin based combination therapy
ACPR	Adequate clinical and parasitological responses
AL	Artemether-lumifantrine
AQ	Amodiaquine
ARF	Acute renal failure
ART	Artemisinin
ART-R	Artemisinin resistance
AS	Artesunate
ASAQ	Artesunate-amodiaquine
ASMQ	Artesunate-mefloquine
ASSP	Artesunate-sulfadoxinepyrimethamine
BGB	Border Guards of Bangladesh
BRAC	Bangladesh Rural Advancement Committee
Bp	Base pair
CAR	Central African Republic
CDC	Centers for Disease Control and Prevention
CHT	Chittagong Hill Tracts
CMCH	Chittagong Medical College Hospital
CMH	Combined Military Hospital
CI	Confidence interval
CQ	Chloroquine
CQR	Chloroquine Resistant
DCA	Delayed clearance assay
DDT	Dichloro Diphenyl Trichloroethane
DGHS	Directorate General of Health Services

DGMS	Directorate General of Medical Service
DHP	Dihydroartemisinin-piperaquine
DNA	Dioxiribonuclic acid
dNTP	Nucleoside triphosphate
DPCT	Delayed parasite clearance time
DRC	Democratic Republic of Congo
G6PD	Glucose-6-phosphate dehydrogenase
GFI	Genotype failure indices
GMS	Greater Mekong Sub-region
GRI	Genotype-resistance index
KAP	Knowledge Attitude and Practice
KARMA	K13 Artemisinin Resistance Multicenter Assessment
ICDDR,B	International Centre for Diarrheal Disease Research, Bangladesh
ICT	Immuno Chromatographic Test
IC50	50% inhibitory concentration
ITN	Insecticide Treated Net
LAMP	loop mediated isothermal amplification
LLIN	Long Lasting Insecticidal Net
LR	Likelyhood Ratio
MDR	Multi-Drug Resistance
MEP	Malaria Eradication Program
MOHFW	Ministry of Health and Family Welfare, Bangladesh
MQ	Mefloquine
NAAT	Nucleic acid amplification test
NEB	New England Biolab
NGO	Nongovernmental organization
NMCP	National Malaria Control Programme
NMEP	National Malaria Elimination Programme

NPV	Negetive Predictive Value
P.	*Plasmodium*
PB	Phenobarbitone
PCR	Polymerase chain reaction
PDR	Peoples Democratic Republic
PE	Pre erythricytic
pfcrt	*Plasmodium falciparum* chloroquine resistance transporter
Pfmdr1	*Plasmodium falciparum* multidrug resistance 1
PPV	Positive Predictive Value
PQ	Primaquine
PRRD1	Parasite reduction ratio 1 day after treatment initiation
QN	Quinine
QT-NASBA	Quantitative nucleic acid sequence-based amplification
RBC	Red blood cell
RDT	Rapid Diagnostic Test
RT PCR	Real time polymerase chain reaction
RFLP	Restriction fragment length polymorphism
SEA	South East Asia
SEARO	WHO Regional Office for South-East Asia
SD	Standard Deviation
SnM-PCR	Semi-nested Multiplex Polymerase chain reaction
SNP	Single-nucleotide polymorphism
SP	Sulfadoxine-pyrimethamine
ssr-RNA	Small subunit ribosomal RNA
TBE	Tris-Borate EDTA
TMDs	Transmembrane domains
TMI	Trophozoite Maturation Inhibition
UN	Unites Nations

Abstract

Despite achieving a trend of reduction in global morbidity and mortality from malaria since beginning of 21st century the parasitic disease continues to have a devastating impact on people's health and livelihoods in Asia and Africa. *Plasmodium falciparum* (*pf*), responsible for most malaria-related deaths among all 5 parasite species, has a long tradition of acquiring resistance against anti-malarial drugs. Development of *pf* resistance to artemisinin, the world's most efficient antimalarial drug, is now threatening the achievements of global malaria control and elimination programmes. Armed forces' personnel in South East Asia and Sub-Saharan Africa traditionally remain exposed to this volatile malarial situation as they usually need to operate in malaria endemic areas. Members of Bangladesh Armed Forces operate in endemic areas of both home and Africa because of their deployment in hilly areas at home and peace keeping missions in Africa. Not surprisingly, they need to travel between different endemic regions. Unfortunately, there is no data available on the genetic types of falciparum malaria they suffer from or probable resistant strains they might transmit between different endemic areas they travel. The present study is designed to investigate and compare molecular pattern and epidemiological aspects of anti-malarial drug resistance of *P. falciparum* in members of Armed Forces of Bangladesh working in endemic areas at home and Africa.

A total of 252 'dried blood samples on filter paper' were collected between November 2014 and February 2016, from *P. falciparum* positive Bangladeshi soldiers working in Chittagong Hill Tracts (CHT), Bangladesh and 5 Sub Saharan African Countries namely, Central African Republic (CAR), Democratic Republic of Congo (DRC), Liberia, Mali and Ivory Coast. These samples (94 from Bangladesh and 138 from African countries) were then transported to Microbiology Laboratories of University of Dhaka, where *pf* DNA extraction was done from all of them using QIAamp® DNA Mini Kit (Qiagen GmbH, Germany), following manufacturer's protocol. Plasmodium species was confirmed by a nested PCR following standard protocol with minor modifications. Thereafter, a multiplex

nested PCR followed by restriction fragment length polymorphism (RFLP) method was employed to investigate the presence of chloroquine resistance marker 'K76T mutation' in *P. falciparum* chloroquine resistance transporters (*pfcrt*) gene and lumifantrine and mefloquine resistance marker 'N86Y mutation' in *P. falciparum* multidrug resistance1 (*pfmdr1*) gene. The propeller region of the kelch 13 (*pfk13*) gene in 29 Bangladeshi and 40 African samples was amplified by a nested PCR following a protocol developed by Pasteur Institute, Paris and Cambodia and then sequenced to see markers of artemisinin resistance. Some data on demography, clinical features, epidemiological aspects and Knowledge attitude and practice (KAP) were also collected from the respondent soldiers using a pretested structured questionnaire and a check list. All molecular and epidemiological data were entered and analyzed in statistical software IBM SPSS version 19.

The *P. falciparum* DNA was confirmed in 35 (37.23%) Bangladeshi and 45 (28.48%) African samples. The '*pfcrt* (K76T) mutation' that confers resistance to chloroquine, was detected in 93.10% Bangladeshi and 29.27% African samples. The '*pfmdr1* (N86Y) mutation' that confers resistance to lumifantrine and mefloquine, was detected in 20.69% Bangladeshi and only 2.44% African samples. None of the Bangladeshi samples had mutation in k13 propeller domain. On the other hand, 9 (22.50%) African samples exhibited *pfk13* mutations including 5 non-synonymous and 3 synonymous mutations, reported for the first time. All of these new non-synonymous mutations namely A617P, Y616F, S491F, N458k and Y616F and synonymous mutations namely F614F, I616I and K503K were found in samples from D R Congo. Mutations F614F and A617P were detected in a single isolate and S491F was detected in two of the isolates. The most common African mutation A578S was detected in a sample from Ivory Coast. None of the *pfk13* mutations, so far recognized to be associated/candidate or validated as artemisinin resistance marker by World Health Organization (WHO), was detected in this study. While analyzing clinical data it was found that, duration of fever, number of fever episodes, failure of drug, referral and length of hospital stay were associated with the 1st line antimalarial drug used ($p < 0.05$). Epidemiological data in this study revealed a yearly

incidence of 180 cases of malaria per thousand population under mefloquine prophylaxis in Liberia, although variables like duration of fever, drug failure and mutation in *pfmdr1* were not associated with this prophylaxis in all the study areas. While analyzing data on knowledge attitude and practice (KAP) it is found that troops under mefloquine prophylaxis were more confident on the efficacy of this prophylactic drug ($P=0.00$). Majority (92%) of the troops could identify mosquito bite as the route of malaria transmission while 62.30% chose bed net as the most important preventive measure against malaria. The correctness of their understanding about malaria transmission, cause of resistance and preventive priorities, was associated with their source of information ($p < 0.05$). More than 90% of the troops used bed net as a protective measure although only 19% of them used insecticide treated net. Regular use of protective clothing to prevent mosquito bite by the soldiers in different study areas varied between 70% and 95%. A routine practice of outdoor mosquito-cidal spray was reported by more than 80% troops working in different countries. Preventive behavior of the troops like use of protective clothing, insecticide treated bed net and mosquito repellent were found to be associated with their source of information ($p < 0.05$).

This study, first of its kind to be conducted in members of Bangladesh Armed Forces deployed in malaria endemic areas of both Bangladesh and Africa, attempted to investigate and compare molecular and epidemiological pattern of antimalarial drug resistance. It revealed 5 new non-synonymous and 3 synonymous mutations in *pfk13* gene found in its African samples. Further study is required to see the relationship of these new mutations with delayed parasite clearance and eventually artemisinin resistance. Further study is also required to examine the potential transportability of drug resistant strains of *pf* malaria between endemic areas.

1.0 Introduction

Malaria is a life-threatening disease caused by *Plasmodium* parasites that are transmitted to people through the bites of infected female anopheles mosquitoes. Among 5 parasite species *Plasmodium falciparum* is responsible for most malaria-related deaths globally. *Plasmodium* parasites especially *P. falciparum* has a long tradition of acquiring resistance against anti-malarial drugs.

Despite being preventable and treatable, malaria continues to have a devastating impact on people's health and livelihoods around the world. According to World Health Organization (WHO), an estimated 219 million cases of malaria occurred worldwide in 2017, compared to 239 million cases in 2010. Estimated number of deaths were 435,000 in 2017 globally as compared to 607,000 in 2010. Children aged under 5 years are the most vulnerable groups affected by malaria. In 2017, they accounted for 61% (266,000) of all malaria deaths worldwide (WHO, 2018[a]). Figure 1.1 shows countries with indigenous malaria cases in 2000 and their status by 2017 as depicted by different colours.

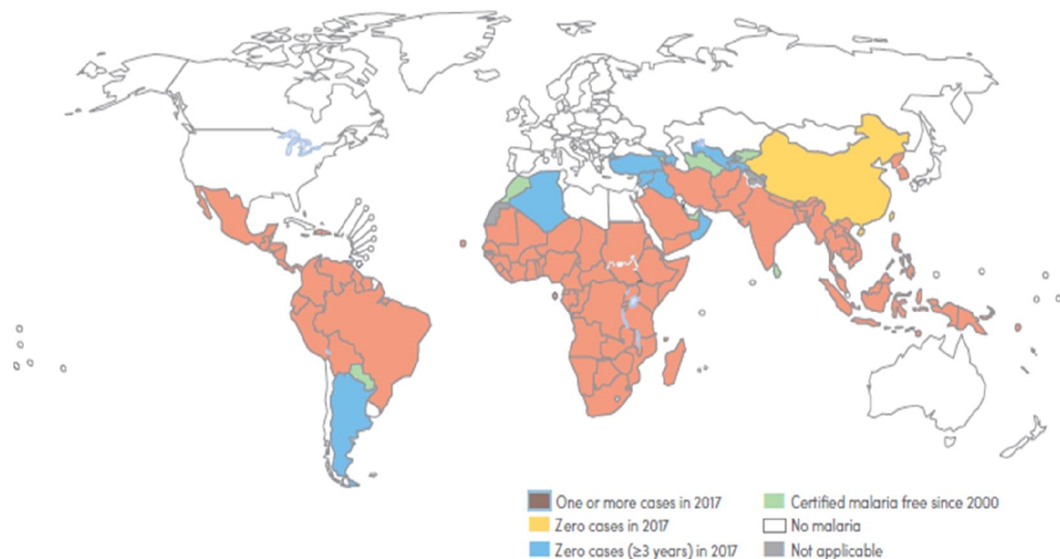

Figure 1.1. Countries with indigenous cases in 2000 and their status by 2017 Countries with zero indigenous cases over at least the past 3 consecutive years are considered to be malaria free. (WHO, 2018[b]).

WHO African Region accounted for the largest proportion of the malaria cases in 2017 (200 million or 92%), followed by the WHO South-East Asia region with 5% of the cases and the WHO Eastern Mediterranean region with 2%. Fifteen countries in sub-Saharan Africa and India carried almost 80% of the global malaria burden. Five countries accounted for nearly half of all malaria cases worldwide: Nigeria (25%), Democratic Republic of the Congo (11%), Mozambique (5%), India (4%) and Uganda (4%) (WHO, 2018[a]).

Among the deaths, almost 80% occurred in 17 African countries along with India. Seven of these countries accounted for 53% of global malaria deaths: Nigeria (19%), Democratic Republic of the Congo (11%), Burkina Faso (6%), Republic of Tanzania (5%), Sierra Leone (4%), Niger (4%) and India (4%). However, a remarkable decline in number of malaria deaths compared with 2010 has been observed in the WHO regions of South-East Asia (54%), Africa (40%) and the Eastern Mediterranean (10%) (WHO, 2018[a]).

Malaria continues to be one of the major public health problems in Bangladesh. More than 13 million people are still at risk of malaria. Prevalent plasmodium species are *P. falciparum* (89%) and *P. vivax* (11%).

The number of malaria cases in Bangladesh fluctuates seasonally. The majority of these cases occur in the thirteen districts close to and/or bordering India and Myanmar, namely, Kurigram, Sherpur, Mymenshingh, Netrokona, Sunamganj, Sylhet, Habiganj, Maulavibazar, Chittagong, Cox's Bazaar, Khagrachari, Rangamati and Bandarban. Three hill districts (Bandarban, Khagrachari and Rangamati) and Cox's Bazar report the highest incidence of malaria within the country. Figure 1.2 shows malaria endemic districts of Bangladesh. Gradient of endemicity is represented by colours.

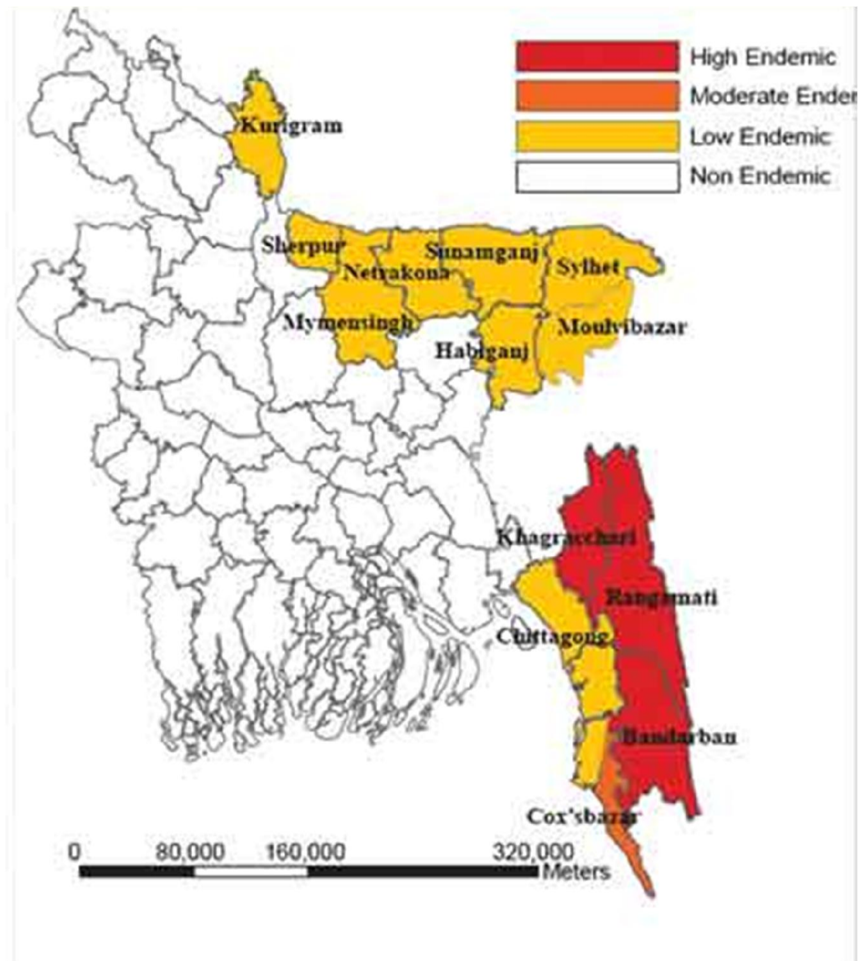

Figure 1.2. Map of Bangladesh showing malaria endemic districts

Maude *et al.* (2012) analyzed malaria screening data from 22,785 in-patients in Chittagong Medical College Hospital (CMCH), a tertiary level hospital which receives malaria patients from most endemic areas from 1999–2011. From 1999 till 2011, 2,394 malaria cases were admitted, of which 96% harbored *P. falciparum* and 4% *P. vivax*. Infection was commonest in males (67%) between 15 and 34 years of age. Seasonality of malaria incidence was marked with a single peak in *P. falciparum* transmission from June to August coinciding with peak rainfall, whereas *P. vivax* showed an additional peak in February-March possibly representing relapse infections. Since 2007 there had been a substantial decrease in the absolute number of admitted malaria cases. Case fatality in severe malaria was 18% from 2008–2011, remaining steady during this period.

According to National Malaria Control Programme of Bangladesh, population at risk of malaria was 17.52 million in 13 endemic districts with 27,737 cases and 17 deaths in 2016 (Bangladesh Health Bulletin 2017). In World Malaria Report 2018 of WHO, estimated cases of malaria in Bangladesh were 32,900 with 76 deaths. *P. falciparum* was alone responsible for 89% malaria cases. (WHO 2018[a]).

Malaria has shaped the course of history of Armed Forces around the world for centuries. It has always been part of the ups and downs of nations; of wars and of upheavals. Many great warriors succumbed to malaria after returning from the warfront and advance of armies into continents was prevented by malaria. In many conflicts, more troops were killed by malaria than in combat (Malaria Site, 2018).

Malaria has also been a great stimulus for research. In 1885 Charles Laveran, a French army doctor working in the military hospital of Constantine in Algeria observed the parasite for the first time inside the red blood cells of people suffering from malaria. After that, Sir Major Ronald Ross, a British military physician working in the Presidency General Hospital in Calcutta India, finally proved in 1898 that malaria is transmitted by mosquitos (Sherman *et al.,* 1998).

In World War I (1914–1918), British, French, and German armies were immobilized by malaria for 3 years in Macedonia. Nearly 80 percent of the French troops in this area were hospitalized with malaria and the British Army lost over 2,000,000 man-days in a single year (1918). (Malaria Site, 2018).

Many troops had to suffer casualties by inflicted malaria even in World War II. Malaria was the most important health hazard encountered by U.S. troops in the South Pacific during the war where about 500,000 men were infected. Sixty thousand American soldiers died of malaria during the North African and South Pacific campaigns (Byrne, 2008). Even in Vietnam War (1962-1975) US Army had over 40,000 cases of malaria.

In recent history, Malaria was the No. 1 cause of casualties among US troops operating in Somalia (1990-1994). In October 2001, a falciparum malaria epidemic that erupted in Afghanistan claimed 53 lives. When 290 marines went ashore in Liberia in September 2003, 80 contracted malaria. Of the 157 troops who spent at least one night ashore, 69 became infected. In Liberia, over a third of U.S. Marines sent in as military advisors to oversee a civil transition have contracted malaria. (Malaria Site, 2018).

Despite achieving a trend of gradual reduction in malaria morbidity and mortality in last decade, malaria endemic countries including Bangladesh relentlessly faces the challenge of drug resistance in the parasite especially *P. falciparum.*

Antimalarial drug resistance – the ability of the malaria parasite to survive drugs, became a global problem for the first time in the 1960s when the parasite developed resistance to chloroquine (CQ), the then widely-used antimalarial drug. Resistance first emerged in the Greater Mekong subregion and later spread to Africa, triggering a dramatic increase in malaria-related illness and death (WHO, 2014[a]).

The problem of antimalarial drug resistance is compounded by cross resistance, in which resistance to one drug confers resistance to other drugs that belong to the same chemical group or which have similar modes of action. During the past decades, several highly efficacious antimalarial drugs had to be removed from markets after the development of parasite resistance to them (WHO, 2014[b]).

The development of resistance can be considered to occur in two phases. In the first phase, an initial genetic event produces a resistant mutant (de novo mutation); the new genetic trait gives the parasite a survival advantage against the drug. In the second phase, the resistant parasites are selected for and begin to multiply, eventually resulting in a parasite population that is no longer susceptible to treatment (WHO, 2010).

One study suggested that *P. falciparum* in South-East Asia has an inherent propensity to develop drug resistance through genetic mutation. It was shown in microsatellite marker studies that *P. falciparum* resistant to chloroquine or highly resistant to pyrimethamine both originated in South-East Asia and subsequently spread to Africa. The emergence of resistance to mefloquine (MQ) arose rapidly on the western border of Cambodia and on the north-west border of Thailand in the 1980s. Epidemiological studies have since shown that the molecular change that led to mefloquine resistance occurred in multiple, independent events, suggesting that it arose in several different places (WHO, 2010).

P. falciparum resistance to artemisinin– the core compound in the world's most effective antimalarial medicines, was first confirmed in 2008 on the Cambodia-Thailand border and is now present in four countries of the Greater Mekong subregion: Cambodia, Myanmar, Thailand and Viet Nam. If not addressed with appropriate urgency, they could threaten the remarkable progress made since 2000. In April 2013, on World Malaria Day, WHO launched an Emergency response to artemisinin resistance in the Greater Mekong subregion to guide countries in the scale-up and implementation of efforts to eliminate resistant parasites (Chan, 2013).

Several molecular markers of *P. falciparum* resistance have been identified. The K76T allele in the CQ resistance transporter gene (*pfcrt*) is associated with CQ and amodiaquine treatment failure. The Y86 allele of multidrug resistance gene 1 (*pfmdr1*) has been linked with CQ and amodiaquine resistance. The role of dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) mutations in the mechanism of resistance to Sulfadoxine-pyrimethamine (SP) has been well described. The sarco/endoplasmic reticulum Ca^{2+}-ATPase ortholog of *P. falciparum* (*pfatp6*) was suggested to be involved in the mechanism of parasite resistance to artemisinin. Specific point mutations in codons 769, 623 and 431 were associated with artemisinin resistance (Huan *et al.* 2012). Recently detected K13-propeller polymorphism constitutes a useful molecular marker of artemisinin resistance in *P. falciparum* (Ariey *et al.* 2014).

Bangladesh, like all other malaria-endemic countries adjusted treatment guidelines to curve spreading resistance to practically all traditional antimalarials and officially recommend artemisinin-based combination therapies (ACTs) as first-line therapy for the treatment of uncomplicated falciparum malaria. The spread of resistance to artemisinin derivates could very well result in one of the most devastating events in the history of malaria control in the 21st century and potentially endanger all recently initiated malaria elimination efforts. Historical evidence from traditional antimalarials suggests that drug resistance has a tendency to spread westwards from its origins in Southeast Asia (Starzengruber *et al.* 2012). Now, the question is that whether artemisinin resistance has already reached the eastern borders of Bangladesh, the traditional gateway of malaria to the subcontinent.

Few molecular data provide strong evidence that artemisinin resistant falciparum malaria in Myanmar extends across much of Upper Myanmar, including areas close to the Indian border in the northwest. These data, associated with information about the presence of K13 mutant alleles in Bangladesh, advocate for a rigorous molecular survey to be implemented without delay in the Indian subcontinent (Mohon *et al.* 2014).

Unfortunately, very limited data are available on the efficacy of malaria treatments and the current situation of anti-malarial drug resistance in Bangladesh. In addition, only a few studies on the molecular characterization of the local parasite population have been performed.

Members of Bangladesh Armed Forces including paramilitary and armed police men working in the endemic areas of the country constitute one of the most vulnerable group to suffer from malaria casualty since independence. The problem was augmented and complicated when these forces started operating in malaria endemic areas of Africa and other continents in peace keeping missions under United Nations. It is obvious that movement of military units between endemic areas of different continents harboring species of plasmodium having different types of resistance to

antimalarials poses a great deal of threat of transporting resistant strains of parasite between the areas they travel.

Therefore, this study has attempted to examine the pattern of *P. falciparum* resistance to antimalarial drugs including artemisinin in a population that remained under represented but exposed to exceptional malarial situations at home and abroad.

2.0 Review of Literature

Fight against malaria was started in the prehistoric era. Even the first effective treatment came much before the causative parasite and its link with the mosquitoes were identified. It was cinchona bark, picked up by Spanish missionaries in the 16th century from the Quechua Indians in Peru who traditionally used this bark for treating shivering fever. Quinine was isolated from this bark in 1820 (Kyle *et al.* 1974). Two military physicians made the next break through, Charles Laveran (a French army doctor) observed the parasite inside the red blood cells of malaria patient and Sir Ronald Ross (at that time a Major in British Indian Army Medical Corps) identified mosquitoes to transmit malaria to human being. Both of them were awarded with the Nobel Prize in 1907 and 1902 respectively.

Discovery of chloroquine in 1934 by Hans Andersag in Bayer Laboratories, Germany was the next milestone (Krafts *et al.* 2012). Extensive use of chloroquine for treating patients and DDT for elimination of mosquitoes were the major tool for the great effort of eradication of malaria in many countries between 1955 to1969. But the progress was halted to great extent by wide spread development of chloroquine resistance of the prasite. The hope of elimination/ eradication again came into being with the discovery of artemisinin, the most effective antimalarial drug in the history, by a Chinese pharmacologist Tu Youyou who was awarded with the Nobel Prize in 2015. But again unfortunately, *P falciparum* is noticed to develop resistance against this golden drug since 2008 (Dondrop *et al.* 2010).

2.1 Malaria and armed forces

More than any other infectious disease, malaria affected the conduct of military operations in war and in many occasions influenced the outcome. From Napoleon's defensive action at Walcheren, to the Union Army's attempts to take control of the Mississippi River at Corinth and Vicksburg, to the dreadful numbers of malaria casualties suffered by U.S. Marines on the islands of Efate and Guadalcanal during World War II and more recently in Liberia in 2003, malaria has extracted a heavy toll. (Christian *et al.* 2005). In fact, malaria took its toll heavily on all the forces operated in endemic belt in World War I, World War II, Korean War, Vietnam War and also recent conflicts in Afghanistan, Somalia and other territories.

Malaria remains a major cause of military casualties in Southeast Asia. The numerous conflicts in the region have been greatly affected by malaria. Besides guerrilla warfare, refugees and other civilian movements across international borders contribute heavily to the continuing problem of multiple drug-resistant malaria. Drug resistance is an increasing problem with few available prophylactic options (Shanks and Karwacki, 1991).

Malaria appeared to be a menace for members of Bangladesh Armed Forces, paramilitary forces and policemen working in the endemic areas since independence. It caused significant morbidity and mortality among troops deployed in the Chittagong Hill Tracts and is still one of the major health problem of the Bangladesh Army (Hussain *et al.* 1996). Following the 1971 ban of DDT (insecticide) in Bangladesh, malaria cases have increased steadily. Lives lost to malaria was much more than that to bullets.

A study carried out by Hussain *et al.* (2003) in Combined Military Hospital Chittagong, Bangladesh showed that 13.62% hospital admission were due to malaria between 1998 and 2001. Out of 7,005 malaria cases, 54.22% were falciparum, 26.18% were vivax and 12.02% were mixed infections. Most of the cases (66.98%) responded to chloroquine. Among the rest, 11.99% required quinine, 9.79% required artemether and 0.08% required both mefloquine and artemether. Total mortality rate

was 0.30% but it was 9.25% and 6.17% among complicated malaria and cerebral malaria cases. Most of the mortality were due to complicated falciparum malaria and the emergence of drug resistance.

Armed Forces of Bangladesh started joining United Nation's peace keeping missions in 1988. Since then, many of the contingents of Bangladeshi troops have been exposed to worst malaria situation in conflict ravaged African countries. They suffer there and many a time the parasitic infection is detected to be borne to Bangladesh. On the other hand, since independence, units of Bangladesh Armed Forces work by rotation in Chittagong Hill Tracts (CHT) and Cox's Bazar near the eastern borders of Bangladesh, the traditional gateway of drug resistant malaria to the subcontinent. These troops might constitute a group that is under-recognized but playing the role of a 'transmission reservoir' for drug resistant malaria. Unfortunately, there is no data available on this issue.

A study carried out in Combined Military Hospital, Dhaka between 2014 and 2015 shows that 43.24% of the troops suffering from vector borne infections were infected by malaria and 25.52% of the malaria cases borne the infection from Ivory Coast, a Sub Saharan African Country where they were deployed on a UN peace keeping mission (Subrina and Shaburul, 2018).

2.2 Biology of malarial parasite

Malaria parasites are micro-organisms that belong to the genus *Plasmodium*. There are more than 100 species of *Plasmodium*, which can infect many animal species such as reptiles, birds, and various mammals. Four species of *Plasmodium* have long been recognized to infect humans in nature (CDC, 2019).

2.2.1 Classification of malarial parasites of medical importance (Levine *et al.* 1980):

Kingdom: Protista

Sub-kingdom: Protozoa Goldfuss, 1918

Phylum: Apicomplexa

Class: Sporozoa Leuckart

Order: Haemosporida Danilewsky

Family: Plasmodiidae Mesnil

Genus: *Plasmodium* Marchiafava and Celli, 1885

Species: *Plasmodium falciparum* Welch, 1897

***Plasmodium vivax* Grassi and Feletti, 1890**

***Plasmodium ovale* Stephens, 1922**

***Plasmodium malariae* Laveran, 1881**

Grassi and Feletti, 1890

Widespread Species are *P. falciparum* and *P. vivax* and less widespread Species are *P. malaria* and *P. ovale.*

2.2.2 *Plasmodium falciparum*

The structural character of the parasite can be studied in stained preparations (Giemsa stained). The morphology stages seen in the stained slides are-

a) Trophozoite
b) Schizont
c) Gametocyte

a. Trophozoite: The early ring form measures 1.25-1.5 µm in diameter. It consists of a fine and uniform cytoplasmic ring with a nucleus. Schuffner's dots are not seen; instead 6-10 Maurer's dots or clefts are seen (Markell *et al.* 1981).

b. Schizont: The nucleus divides into several masses varying from 8 to 36 in number. (Markell *et al.*1981) The cytoplasm also divides, form many segments arranged around the central pigment mass.

c. Gametocytes: There are two types of gametocytes. These are-

i. Microgametocyte: They are banana or crescent shaped. Broader, shorter and size varies from 8-10 µm by 2-3 µm and are scattered throughout the cytoplasm (Chatterjee, 1980).

ii. Macrogametocyte: The shape is crescentic. They are longer, narrower and size varies from 10-10 µm by 2-3 µm and aggregate like a wreath round the nucleus (Chatterjee, 1980).

Figure 2.1 shows different blood stage parasites of *P. falciparum.*

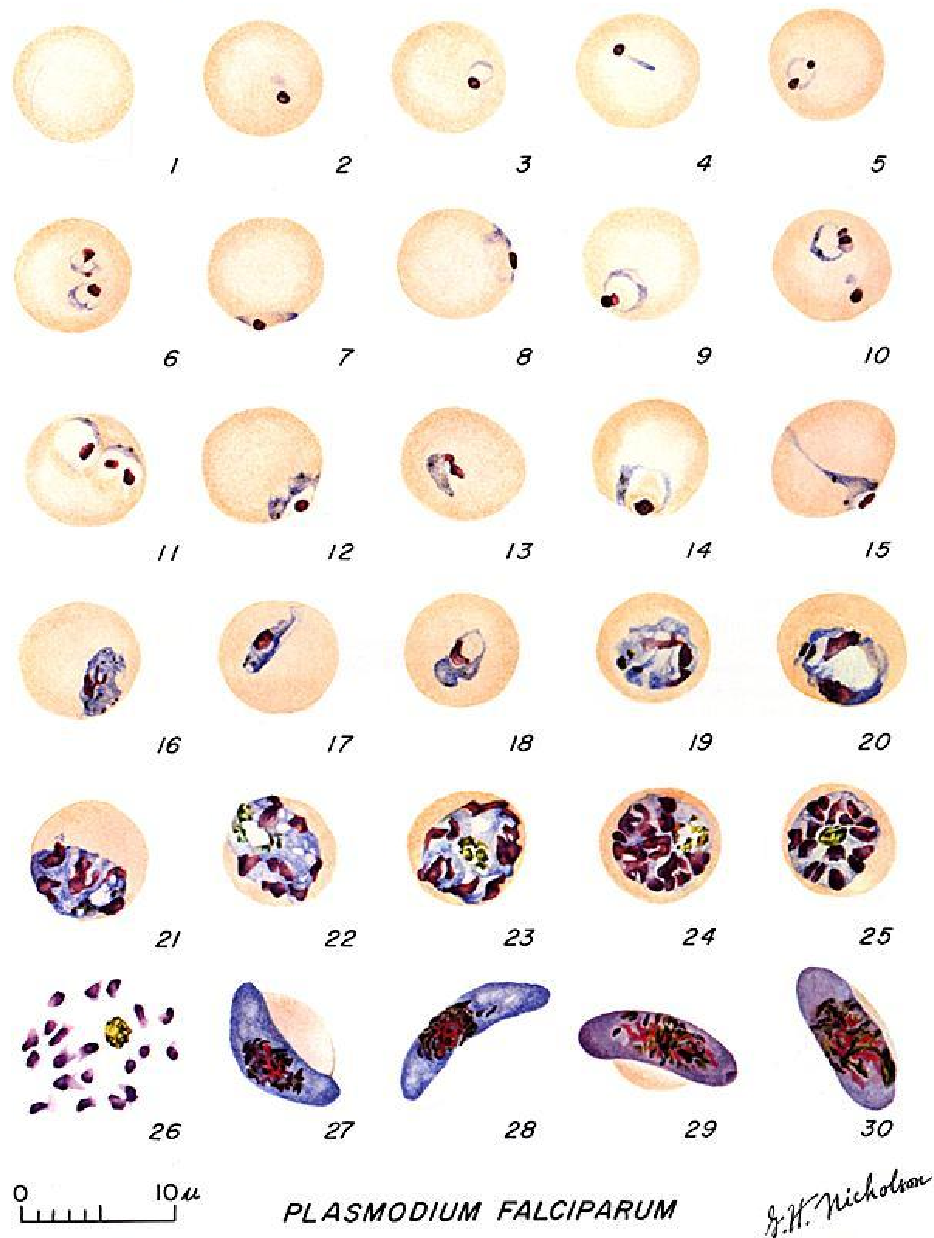

Figure 2.1. *P. falciparum*: blood Stage Parasites: Fig. 1: Normal red cell; Figs. 2-18: Trophozoites (among these, Figs. 2-10 correspond to ring-stage trophozoites); Figs. 19-26: Schizonts (Fig. 26 is a ruptured schizont); Figs. 27, 28: Mature macrogametocytes (female); Figs. 29, 30: Mature microgametocytes (male). (Coatney *et al.* 1971).

2.2.3 Life cycle of *P. falciparum*

Sporozoites contained in the saliva of an infected *Anopheles* mosquito are injected into the blood of a human host when the vector takes a blood meal. The sporozoites are elongate bodies measuring about 11 micrometers in length, with a central nucleus. After circulating in the bloodstream for not more than one hour, the sporozoites enter liver cells probably by way of the Kupffer cells (Schmidt *et al.* 1989). In liver cells, *P. falciparum* parasites grow, multiply and develop directly into schizonts. The schizonts in the liver are referred to as pre-erythrocytic (PE) schizonts. PE schizonts of *P. falciparum* take 5 ½ - 7 days to develop. When mature each measures about 60 micrometer in diameter and contains up to 30000 merozoites. (Schmidt *et al.* 1989) When mature, the schizont and liver cell rupture and the merozoites enter the blood stream. To survive, the merozoites must enter red cells within a few minutes of being released from the schizont Most of the merozoites enter red cells in the sinusoids of the liver. A proportion are phagocytosed and destroyed. Entry of the parasites into red cells starts a cycle in the blood which for *P. falciparum* takes 36-48 hours to complete. The incubation time for *P. falciparum* from infection to attack is 8-11 days (Markell *et al.* 1981). For the life cycle to be continued, the gametocytes need to be ingested by a female Anopheles mosquito in a blood meal. If they are not taken up by a mosquito vector, they die. No Exoerythrocytic schizogony. As a result it doesn't relapse (Chatterjee, 1980).

In the crop of the mosquito, the gametocyte (microgametocyte) rapidly divides into a number of male gametes with motile flagella. Fertilization occurs between the male and female gametes inside the crop of mosquito. The male nucleus fuses with female nucleus and a zygote is formed.

The zygote develops into motile ookinete which penetrate the crop wall of mosquito and form an oocyst. In oocyst, large numbers of sporozoites are formed. Mature sporozoites leave the oocyst and spread to all parts of mosquito, particularly to salivary glands, and wait for transmission into human host during a blood meal by the mosquito (Asna *et al.* 1996). Figure 2.2 shows life cycle of *P. falciparum* in both hosts.

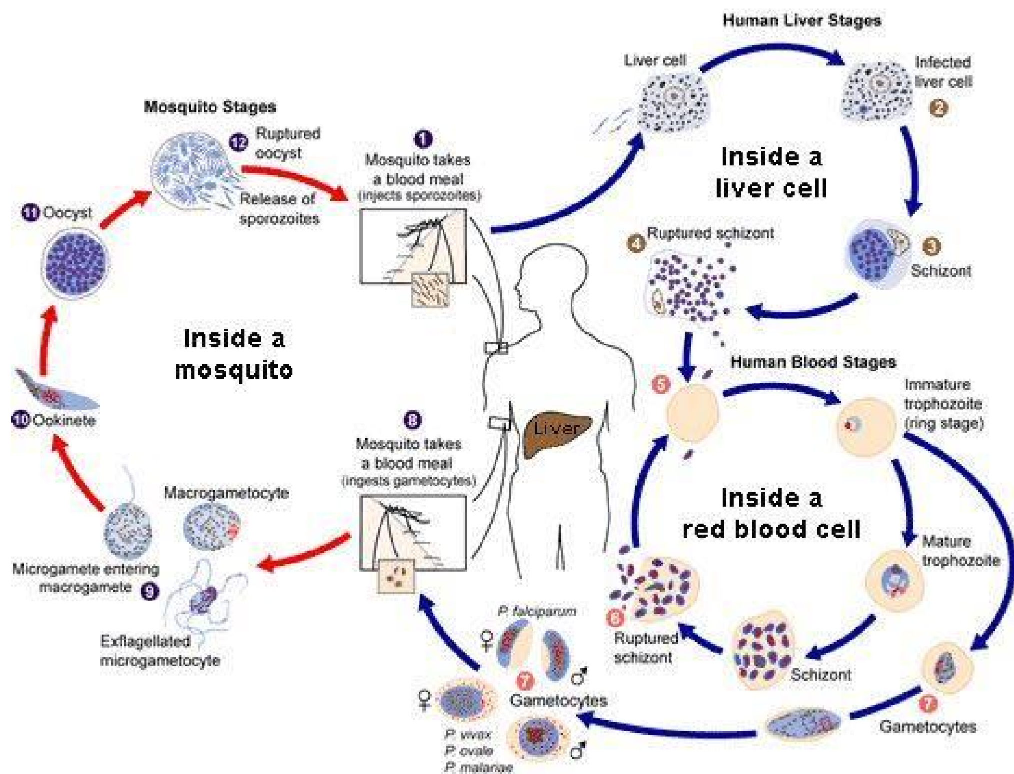

Figure 2.2. Life cycle of *P. falciparum* (CDC 2019)

2.2.4 Clinical features of *P. falciparum* malaria

The classic symptom of malaria is cyclical occurrence of sudden coldness followed by rigor and then fever and sweating lasting four to six hours, occurring every two days in *P. vivax* and *P. ovale* infections, while every three days for *P. malariae*. *P. falciparum* can have recurrent fever every 36–48 hours or a less pronounced and almost continuous fever. For reasons that are poorly understood, but that may be related to high intracranial pressure, children with malaria frequently exhibit abnormal posturing, a sign indicating severe brain damage. Malaria has been found to cause cognitive impairments, especially in children. It causes widespread anemia during a period of rapid brain development and also direct brain damage. This neurologic damage results from cerebral malaria to which children are more vulnerable. Cerebral malaria is associated with retinal whitening, which may be a useful clinical sign in distinguishing malaria from other causes of fever. Signs and symptoms of malaria may also include arthralgia (joint pain), vomiting, anemia

(caused by hemolysis), hemoglobinuria, retinal damage, and convulsions. A falciparum malaria infection should be considered as severe when more than 5% of the red cells become parasitized.

2.2.5 Important complications of falciparum malaria and their pathophysiology

Severe malaria is almost exclusively caused by *P. falciparum* infection, and usually arises 6–14 days after infection. Consequences of severe malaria include coma and death if untreated—young children and pregnant women are especially vulnerable. Splenomegaly (enlarged spleen), severe headache, cerebral ischemia, hepatomegaly (enlarged liver), hypoglycemia, and hemoglobinuria with renal failure may occur. Few most important of those complications are discussed below in brief.

2.2.5.1 Cerebral malaria

Cerebral malaria is the commonest cause of death in falciparum malaria and is due to parasitized red cells and fibrin blocking capillaries and venules in the brain. Extensive microvascular obstruction and impaired perfusion in brain is the critical pathophysiological processes in cerebral malaria. It is found in patients who die in the acute phase of cerebral malaria that, many of the cerebral capillaries and venules are packed tightly with parasitised erythrocytes, whereas other adjacent vessels are not obstructed. The degree of packing and congestion of the cerebral micro vessels with both infected and uninfected red cells is associated significantly with the level of pre-mortem coma and the interval to death. A distinct and highly specific malaria retinopathy occurs in both children and adults with cerebral malaria (WHO, 2014).

2.2.5.2 Anemia

The pathophysiology of the anaemia of falciparum malaria is both complex and multifactorial, and results in a condition which is a major cause of mortality and morbidity in patients, especially children and pregnant women, living in malarial endemic areas. The importance of anaemia as a cause of death in malaria may well be underestimated because of difficulty in diagnosis, especially where parasitaemia may be low and the clinical picture may be confused with other causes of anaemia. Two clinical presentations predominate: severe acute malaria in which anaemia

supervenes, and severe anaemia in patients in whom there have been repeated attacks of malaria. The major mechanisms are those of red cell destruction and decreased red cell production. Potential causes of haemolysis include loss of infected cells by rupture or phagocytosis, removal of uninfected cells due to antibody sensitization or other physicochemical membrane changes, and increased reticuloendothelial activity, particularly in organs such as the spleen. Decreased production results from marrow hypoplasia seen in acute infections, and dyserythropoiesis, a morphological appearance, which in functional terms results in ineffective erythropoiesis. The role of parvovirus B19 as a possible cause of bone marrow aplasia in a few cases is postulated. Finally, there is now evidence which points to genetic factors, HLA associated, which may protect against the development of malarial anaemia and which has become common in areas endemic for malaria.

2.2.5.3 Renal failure

Acute renal failure (ARF) is seen mostly in Plasmodium falciparum infection. Malarial ARF is commonly found in non-immune adults and older children with falciparum malaria. Occurrence of ARF in severe falciparum malaria is quite common in Southeast Asia and Indian subcontinent where intensity of malaria transmission is usually low with occasional microfoci of intense transmission. Since precise mechanism of malarial ARF is not known, several hypotheses including mechanical obstruction by infected erythrocytes, immune mediated glomerular and tubular pathology, fluid loss due to multiple mechanisms and alterations in the renal microcirculation, etc. have been proposed.

2.3 Diagnosis of malaria

Early and accurate diagnosis of malaria is essential for both effective disease management and surveillance. High-quality diagnosis is important in all settings as misdiagnosis can result in significant morbidity and mortality.

2.3.1 Symptomatic diagnosis

Areas that cannot afford even simple laboratory diagnostic tests often use only a history of subjective fever as the indication to treat for malaria. Using Giemsa-stained blood smears from children in Malawi, one study showed that when clinical predictors (rectal temperature, nail bed pallor and splenomegaly) were used as treatment indications, rather than using only a history of subjective fevers, a correct diagnosis increased from 21% to 41% of cases, and unnecessary treatment for malaria was significantly decreased.

2.3.2 Microscopy

The most economic, preferred and reliable diagnosis of malaria is the microscopic examination of blood films. Malaria microscopy allows the identification of different malaria-causing parasites (*P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*), their various parasite stages, including gametocytes, and the quantification of parasite density to monitor response to treatment. Microscopy is the method of choice for the investigation of malaria treatment failures. Giemsa is the classical stain used for malaria microscopy, and diagnosis requires examination of both thin and thick films from the same patient. Light microscopy is the diagnostic standard against which other diagnostic methods have traditionally been compared. (WHO, 2019). Figure 2.3 shows microscopic appearances of different stages of 4 *plasmodium* species.

Species / Stages	P. Falciparum	P. Vivax	P. Malariae	P. Oval
Ring Stage				
Trophozoite				
Schizont				
Gametocyte				

Figure 2.3. Microscopic appearances of different stages of 4 *plasmodium* species (Jan *et al*. 2018)

2.3.3 Rapid Diagnostic Tests (RDT)

RDTs are relatively simple to perform and interpret, they rapidly provide results, require limited training, and allow for the diagnosis of malaria at the community level. RDTs have the potential to greatly improve the quality of management of malaria infections, especially in remote areas with limited access to good quality microscopy services. Malaria RDTs detect specific antigens (proteins) produced by malaria parasites that are present in the blood of infected individuals. Some RDTs detect a single species (either *P. falciparum* or *P. vivax*), some detect multiple species (*P. falciparum*,*P. vivax*, *P. malariae* and *P. ovale*) and some further distinguish between*P. falciparum* and non-*P. falciparum* infection, or between specific species. Blood for the test is commonly obtained from a finger-prick and results are available within 15–30 minutes. Though there are variations among the more than 200 malaria RDT products on the market, the principles of the tests are similar (WHO, 2019).

2.3.4 Molecular detection of malaria

Microscopy and RDTs are the primary choices for diagnosing malaria in the field, but neither method is capable of detecting low density malaria infections, common in both low and high transmission settings. Nucleic acid amplification tests (NAATs) enable sensitive detection of low density malaria infections (below 1 parasite/µL). Polymerase chain reaction (PCR) – including nested (n), quantitative (q) or real-time reverse transcription (RT PCR), loop mediated isothermal amplification (LAMP), and quantitative nucleic acid sequence-based amplification (QT-NASBA) – are among the key NAATs developed to detect malaria (WHO, 2019).

2.3.5 Molecular detection of *P. falciparum*

Genus- and species-specific sequences are present within the small subunit 18S rRNA genes of the four human malaria parasites. Oligonucleotide primer pairs specific to each species are used for specific amplification by the Polymerase Chain Reaction to detect each malaria species.

Snounou *et al.* (1993) conducted a study on the identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infections. Oligonucleotide primer pairs specific to each species were designed for specific amplification by the Polymerase Chain Reaction (PCR), to detect each malaria species. Blood samples obtained from 196 patients attending a malaria clinic in Trad province (Thailand) were analyzed. Detection and identification of the parasites, solely by electrophoretic analysis of the PCR products, has proven to be more sensitive and accurate than by routine diagnostic microscopy.

Sing *et al.*, (1996) used a modified nested polymerase chain reaction (PCR) method for detection of *P. falciparum*, *P. vivax* and *P. malariae* in Malaysia. Finger-prick blood samples from 46 hospital patients and 120 individuals living in malaria endemic areas were spotted on filter papers and dried. Higher malaria prevalence rates for both clinical (78·2%) and field samples (30·8%) were obtained with the nested PCR method than by microscopy (76·1% and 27·5%, respectively). Nested PCR was more

sensitive than microscopy in detecting mixed *P. falciparum* and *P. vivax* infections, detected 5 more malaria samples than microscopy on the first round of microscopical examination, and detected malaria in 3 microscopically negative samples. Nested PCR failed to detect parasite DNA in 2 microscopically positive samples, an overall sensitivity of 97·4% compared to microscopy.

Cox-Singh *et al.* (1997) worked on increased sensitivity of malaria detection by nested polymerase chain reaction using simple sampling and DNA extraction. In order to study malaria in all geographical locations it is important that specimen collection and DNA extraction for PCR be kept simple. They reported a method for extracting DNA from dried blood spots on filter paper which is capable of detecting one *P. falciparum* and two *P. vivax* parasites/microliter of whole blood by nested PCR without compromising the simplicity of specimen collection or DNA extraction.

Ciceron *et al.* (1999) made a study to development of a *Plasmodium* PCR for monitoring efficacy of antimalarial treatment. They reported a highly sensitive and nonradioactive PCR method for the detection of the four species of parasite causing human malaria. *Plasmodium*-specific primers corresponding to the small-subunit rRNA genes of the malaria parasite were used, and a 291-bp fragment was amplified. Our results showed a high specificity for the four human *Plasmodium* species, and we were able to detect one parasite in 50 ml of whole blood. The responses of 12 patients infected with *P. falciparum* to antimalarial therapy were monitored by PCR diagnosis and examination of thick blood film for at least 20 min by an experienced microscopist. For one patient this study allowed early diagnosis of therapeutic failure, confirmed 7 days later by examination of the thick blood film. A total of 134 samples were examined; 94 were positive by PCR, and among these 68 were positive by thick blood film examination. The sensitivity of the thick blood film was 72.3% compared to PCR and 60.7% compared to dot blot hybridization.

Siribal *et al.* (2004) evaluated species specific nested PCR in testing 40 blood samples from malaria patients admitted during July-September, 2003, at the Hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University, Thailand.

Parasite DNA of each blood sample was extracted and purified by QIAamp. DNA mini \kit. Nested PCR was performed using genus-specific primers for the first PCR cycle and species-specific primer for the second cycle. Thin and thick smears were also made, stained with Giemsa, and examined by expert microscopists. Only one of 40 samples (2.5%) was identified as *Plasmodium malariae* infection by both microscopy and nested PCR. Twenty blood samples (50%) were identified as *P. falciparum* infections by both methods. However, 19 blood samples (47.5%) were reported as Plasmodium vivax infections by microscopic methods, whereas nested PCR could detect a mixed infection of *Plasmodium vivax* and *P. falciparum* in one sample taken from a young girl with 8 ameboid trophozoites of *P. vivax* per 200 white blood cells. These results demonstrated that the nested PCR assay surpasses microscopy and also offered a clear advantage in the detection of mixed infections, which is important not only for successful medical treatment, but also for the study of malaria epidemiology.

Aslan *et al.* (2007) evaluated the performance of this method in the diagnosis of malaria suspected cases in Turkey by comparing to microscopy of the blood smears: blood samples were obtained from 114 patients with malaria symptoms, including fever and/or chills lasting for several days, before starting treatment. Thin and thick blood smears were prepared immediately in the region of specimen collection. After isolation of DNA from blood samples, DNA was amplified by PCR and digested by restriction enzyme AluI. The obtained fragments were analyzed by agarose gel electrophoresis. The number of parasites in the thick and thin smears of the blood samples was evaluated microscopically after staining by Giemsa and results were compared by PCR results. Among 114 plasmodium positive cases detected by microscopy, 100 were also detected by PCR. There were 14 false negatives and no false positive by PCR. Compared to microscopy, the sensitivity, specificity and Positive Predictive Value (PPV) of PCR were determined as 76%, 100% and 100%, respectively.

Parajuli *et al.* (2009) carried out a comparative evaluation of microscopy and polymerase chain reaction (PCR) for the diagnosis in suspected malaria patients in Nepal. Microscopy has been the most common technique for the diagnosis of malaria in Nepal. During July-August 2007, blood samples were collected in glass slides and on filter papers from suspected malaria cases of Kanchanpur, Jhapa and Morang Districts. Sample transportation and storage was done using standard protocol. Microscopy was done at the heath posts in the district in Nepal while Nested PCR using previously standardized primers was carried out at Mahidol University. Among 824 malaria suspected cases, 19.2% (157) were laboratory confirmed as malaria cases (*P. vivax* 10.9%, *P. falciparium* 7.7% and 0.4% were of mixed infection) by microscopy. The parasite count range was detected as 320-25020 parasites/µl. Among total 132 samples (114 microscopic positive, 18 negative) were processed for nested PCR. Among microscopic positive samples with increase of the parasitaemia/µl of the blood, the rate of detection by PCR (75.4%) was increased though the PCR failed to detect 2 cases having the parasitaemia 5000-15000/µl of blood however 4 microscopic negative cases were detected as *P. vivax* infection. Among the microscopy positive samples for *P. falciparum*, 3 were found *P. vivax* and 2 were found as mixed infection of Pv and Pf and 6 *P. vivax* were found positive for *P. falciparum* by PCR. Two microscopy positive samples for mixed infection were found be positive for one Pv and one Pf by PCR.

Ogbolu *et al.* (2012) evaluated the usefulness of polymerase chain reaction in the diagnosis of malaria in Nigeria. Microscopy has been the most common technique for the diagnosis of malaria in south western Nigeria. Using microscopy as reference gold standard, patients comprising 120 males and 330 females with age ranging between less than 1 and 60 samples were used. In all, about 255 (56.7 %) of the samples were positive for microscopy, while 75 (16.7 %) with high parasitaemia on microscopy were positive for PCR analysis. The study concluded that PCR for diagnosis of malaria had sensitivity of 29.4% and specificity of 100% using crude method of DNA extraction while the use of DNA extraction kit had sensitivity of 90.2% and specificity of 100%, hence effort should be geared towards increasing the sensitivity and reduce the cost of doing the test in low resource country like Nigeria.

Ojurongbe *et al.* (2013) carried out a study in Nigeria to compare the performance of clinical diagnosis and three laboratory diagnostic methods (thick film microscopy (TFM), rapid diagnostic test (RDT), and polymerase chain reaction (PCR)) for the diagnosis of *P. falciparum*. Depending on clinical criteria, 217 children were recruited into the study out of which 106 (48.8%) were positive by TFM, 84 (38.7%) by RDT, and 125 (57.6%) by PCR. Using a composite reference method generated from the three diagnostic methods, 71 (32.7%) patients were found to be truly infected and 90 (41.5%) truly uninfected, while 56 (25.8%) were misidentified as infected or noninfected. When each of the 3 diagnostic methods was compared with the composite reference, PCR had sensitivity of 97.3%, specificity of 62.5%, positive predictive value (PPV) of 56.8%, and negative predictive value (NPV) of 97.8%; microscopy had sensitivity of 77.2%, specificity of 72%, PPV of 66.9%, and NPV of 81.1%, while RDT had sensitivity of 62.3%, specificity of 87.4%, PPV of 67.7%, and NPV of 84.5%. PCR test performed best among the three methods followed by TFM and RDT in that order.

In India, Saha *et al.* (2017) evaluated smear microscopy, rapid diagnostic test (RDT), and polymerase chain reaction (PCR) for diagnosis of malaria using Bayesian latent class analysis. The magnitude of malaria was 17.7% (95% confidence interval: 12.5%–23.9%) among the study subjects. The sensitivity of microscopy was 63%, but it had very high specificity (99.4%). Sensitivity and specificity of RDT and PCR were high with RDT having a marginally higher sensitivity (94% vs. 90%) and specificity (99% vs. 95%). On comparison of likelihood ratios (LRs), RDT had the highest LR for positive test result (175) and the lowest LR for negative test result (0.058) among the three tests.

In Equatorial Guinea Berzosa *et al.* (2018) analyzed the performance of microscopy and RDTs, the two main techniques used for the diagnosis of malaria, compared to semi-nested multiplex PCR (SnM-PCR). A total of 1724 samples, tested by microscopy, RDT, and SnM-PCR were analysed. Among the negative samples detected by microscopy, 335 (19.4%) were false negatives. On the other hand, the negative samples detected by RDT, 128 (13.3%) were false negatives based on PCR.

This finding is important, especially since it is a group of patients who did not receive antimalarial treatment. Owing to the high number of false negatives in microscopy, the researchers felt it necessary to reinforce training in microscopy.

In malaria endemic North East Indian provinces Ahmed *et al.* (2018) evaluated malaria diagnostic performance through microscopy and rapid diagnostic test (RDT) while accuracy was evaluated by nested PCR. Of 1173 symptomatic malaria suspected patients, 15.6% (183/1173) patients were diagnosed as malaria positive by RDT and 67.94% cases (53/78) with microscopy. Of 183 malaria-positive patients, 42.62% (78/183) were diagnosed with *P. falciparum* and 84.61% (66/78) further confirmed to be *P. falciparum* positive by nested PCR. High sensitivity (97.9%) and low specificity (2.03%) of the RDT and high sensitivity (99.1%) and low specificity (0.9%) in microscopy against nested PCR results was statistically significant ($P < 0.05$). Epidemiological comparisons expressed highest incidences in Manipur (51.11%) followed by Meghalaya (48.93%) and Assam (35.16%). Overall incidence rate among the genders was observed to be higher in males than in females.

2.4 Malaria and drug resistance

Emergence of resistance to antimalarial drugs has become a major hurdle in the successful treatment of the infection, and has contributed significantly to global malaria-related mortality (WHO, 2010[b]). Drug pressure has been identified as one of the key factors for the emergence and spread of resistance. The contribution of the extensive use and misuse of antimalarial drugs to the selection of resistant parasites became particularly evident during the Global Malaria Eradication campaign, launched by WHO in 1955. The first reports confirming *P. falciparum* resistance to CQ came in the early 1960s almost simultaneously from South-East Asia and South America where direct or indirect mass drug administration (MDA) had been implemented. Similar approaches were very limited in Africa, where *P. falciparum* resistance to CQ was first reported from the eastern region in the late 1970s and spread progressively to the west (Alessandro *et al.* 2001). Figure 2.4 displays global distribution of resistance to chloroquine, sulfadoxine-pyrimethamine and mefloquine as in 2004.

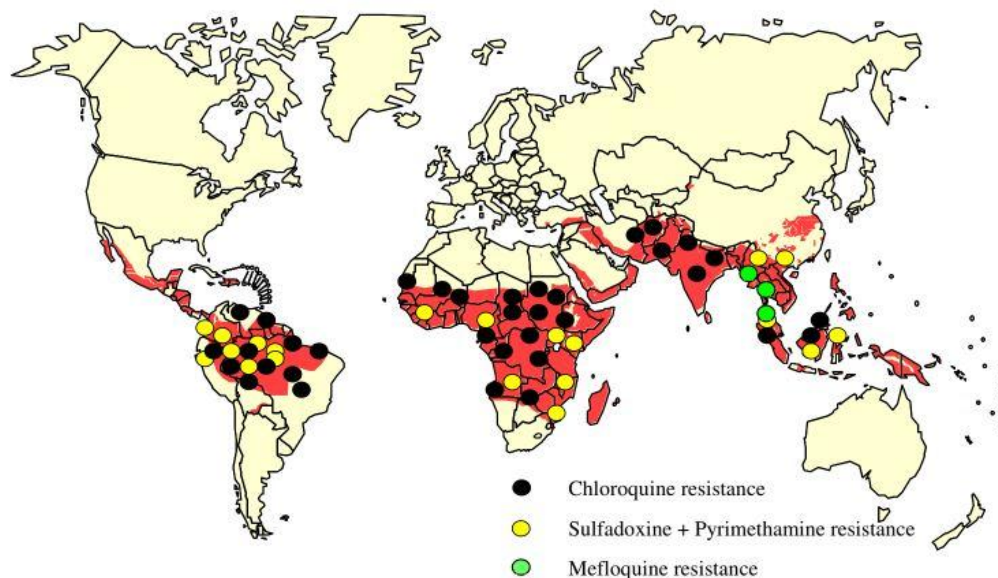

Figure 2.4. Global distribution of resistance to three common anti-malarial drugs in 2004 (Parek, 2004)

2.4.1 Development of resistance

Malaria parasite is well known for its frequent, de novo mutations, mostly single, and sometimes multiple. In the presence of heavy infection and inadequate drug levels, the resistant mutations survive and propagate. Development of resistance requires a high grade of parasitemia, coupled with low or inadequate drug levels. Most cases of resistance have emerged out of SE Asia region. This region is known for low transmission and low immunity that lead to high parasitemia. One study also suggested that *P. falciparum* in South-East Asia has an inherent propensity to develop drug resistance through genetic mutation (Malaria Site, 2019[b]). Figure 2.5 shows the pathway of development of resistance in malaria parasite.

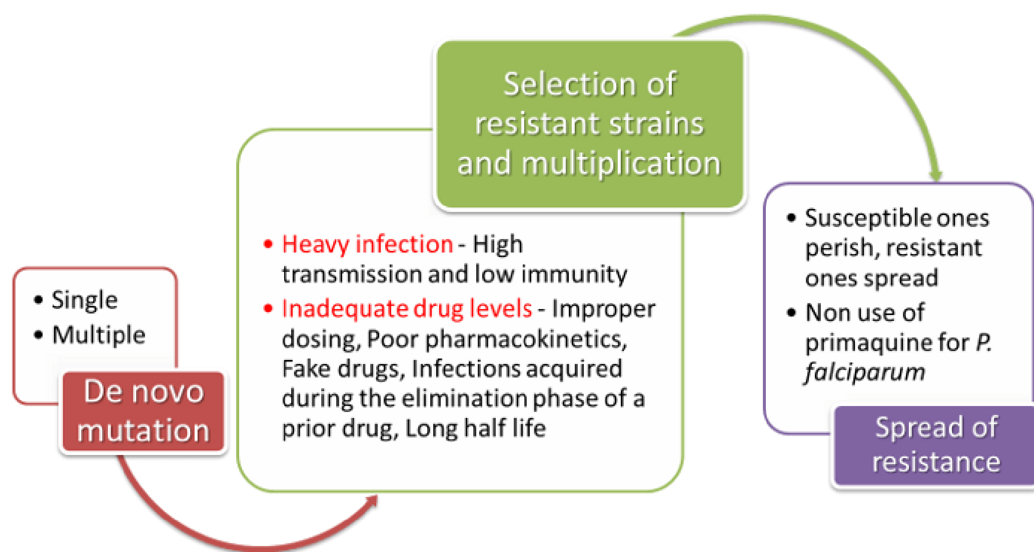

Figure 2.5. Development of resistance in malaria parasite (Malaria Site, 2019[b])

2.4.2 Mechanisms of resistance

The biochemical mechanism of resistance has been well understood in cases of chloroquine, the antifolates, and atovaquone. The chloroquine-resistant strains of *P. falciparum* tend to accumulate the drug less efficiently than the sensitive ones. Polymorphism in the *pfcrt* (*P. falciparum chloroquine resistance transporter*) gene, particularly the one amino acid change, K76T, located in the first transmembrane domain, has been found consistently in chloroquine-resistant *P. falciparum* parasites. This critical K76T mutation could possibly alter the selectivity of CRT such that chloroquine more efficiently exits the food vacuole. Another mutation could be at the *pfmdr1* (*P. falciparum multi drug resistance 1*) gene encoding for the transporter for importing solutes into the food vacuole, including the drugs mefloquine and halofantrine (Boland, 2001). Parasite genes having mutations, established to be responsible for anti-malarial drug resistance are listed in table 2.1.

Table 2.1. List of parasite genes to have mutations responsible for drug resistance (Malaria Site, 2019[b])

Drug	Genes to have mutations responsible for drug resistance
Chloroquine	*pfcrt*, the key amino-acid change required to allow resistance to emerge has been identified as K76T), *pfmdr1*
Quinine	*pfcrt*, *pfmdr1*,and *pfnhe1* (*P.falciparum* sodium/proton exchanger 1)
Amodiaquine	*pfcrt*, *pfmdr1*
Mefloquine	*pfmdr1*
Piperaquine	*pfcrt*
Lumefantrine	*pfcrt*, *pfmdr1*
Atovaquone	Single nucleotide polymorphisms in the cytochrome *b* gene
SP	Point mutations in *pfdhfr* and *pfdhps*
Artemisinin	*pfk13* (Pf kelch protein gene on chromosome 13)

2.4.2.1 The chloroquine resistance transporter (*pfcrt*) and its functional role

The *pfcrt* gene is situated on chromosome 7 of *P. falciparum* DNA and encodes a transport protein in the vacuolar membrane. This gene plays a major role in determining the phenotype of chloroquine resistance drug of 4-Aminoquinolines family, when lysine is replaced at codon 76 by threonine. This mutation is associated with different sets of mutations at other codons, most commonly Cys72Ser, Met74Ile, Asn75Glu, Ala220Ser, Gln271Glu, Asn326Ser, Ile356Thr and Arg371Ile, although the specific set of accompanying mutations depends on the geographical setting. Some chloroquine-resistant isolates have shown cross-resistance with amodiaquine both in vivo and in vitro. *pfcrt* and *pfmdr1* alleles interact to yield different levels of resistance to chloroquine and amodiaquine. The *pfcrt* mutations at codons 72–76 observed in South America are associated with high levels of amodiaquine resistance, whereas *pfcrt* mutations in South-East Asia and Africa are linked to greater resistance to chloroquine and moderate resistance to amodiaquine. This difference may be due to the extent of previous use of amodiaquine in different regions. Amodiaquine resistance may also be modulated by the *pfmdr1* mutations Asn86Tyr and Asn1042Asp (Sá *et al.,* 2009).

Pfcrt is predicted to contain 10 transmembrane domains (TMDs) and to be orientated in the DV membrane with the N and C termini extending into the cytosol. Depending on the strain, *Pfcrt* can contain anywhere between 4 and 10 mutations, with a total of 32 polymorphic residues identified to date. However, one mutation—the substitution of the lysine at position 76 for threonine (K76T)—has been found in almost all CQR field isolates. This results in the efflux of chloroquine from the vacuole 50 times faster than the parasite without mutation. Figure 2.6 shows a scheme display of the location of the ~100 Kb segment present in the seventh chromosome of *P. falciparum* harboring the transporter genes, *pfcrt* and *var* gene. Figure 2.7 displays a Predicted protein structure of *pfcrt.*

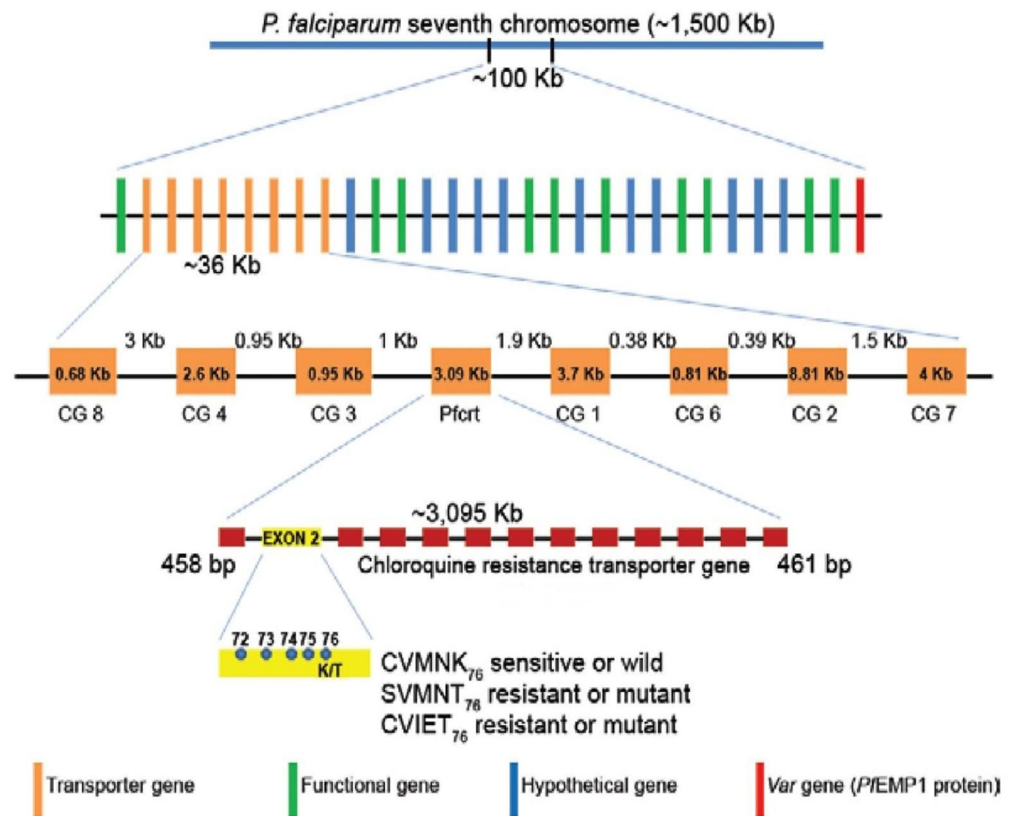

Figure 2.6. Location of the ~100 Kb segment present in the seventh chromosome of *P. falciparum* harboring the transporter genes, *pfcrt* gene. Further ~36 Kb segment is highlighted encompassing the eight transporter genes including the *pfcrt* gene and a more schematic view of the *pfcrt* gene with its 13 exons and the K76T mutation is highlighted. The five amino acids present from 72 76 position in exon 2 characterize the resistant (CVIET and SVMNT) and sensitive (CVMNK) chloroquine resistance *Pfcrt* haplotypes (Awasthi *et al.* 2013).

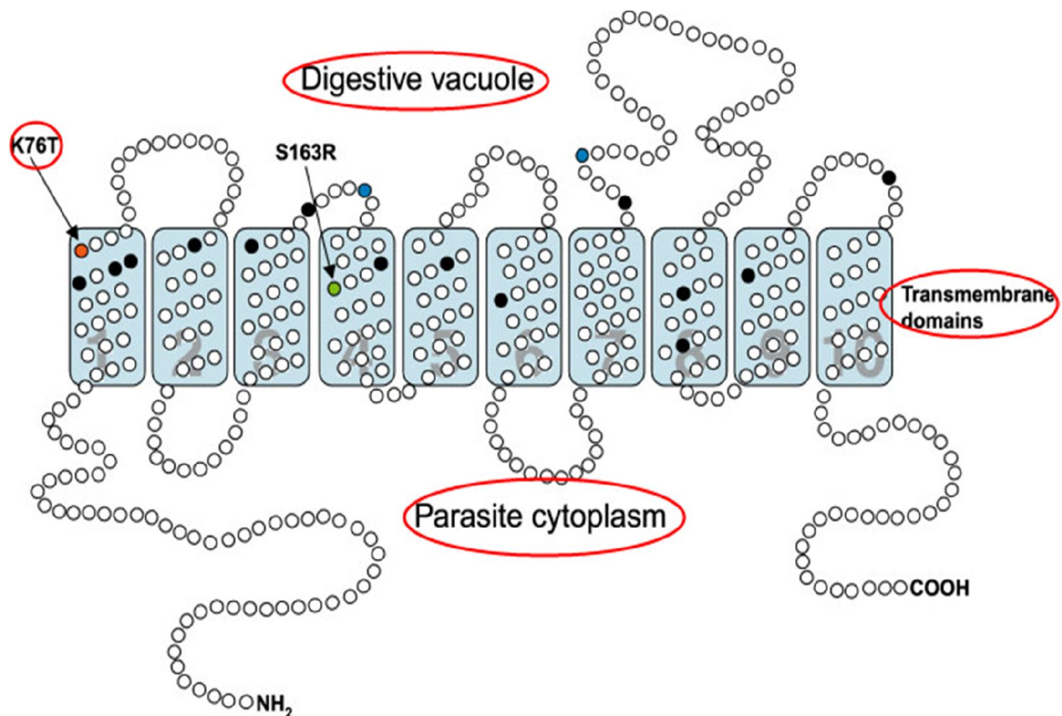

Figure 2.7. Predicted protein structure of *pfcrt*. *Pfcrt* is postulated to possess 10 transmembrane helices, with the N- and C-termini extending into the parasite cytoplasm. Black and red filled circles indicate the positions of mutations published from full-length *pfcrt* cDNA sequences identified in CQR parasites from field samples (Fidock *et al.* 2000, Chen *et al.* 2003, Nagesha *et al.* 2003 and Durrand *et al.* 2004).

2.4.2.2 The multidrug resistance 1 protein (*pfmdr1*) and its functional role

The *pfmdr1* gene, which is situated on chromosome 5 of *P. falciparum* DNA and codes for the P-glycoprotein homologue 1, responsible for multidrug resistance. The mutations to *pfmdr1* that have been associated with chloroquine resistance include Asn86Tyr, Tyr184Phe, Ser1034Cys, Asn1042Asp and Asp1246Tyr. The *pfmdr1* gene has also been implicated in resistance to amino-alcohols drug family and to artemisinins. *pfmdr1* has been predicted to have 12 transmembrane proteins. The substitution of the Asparagine at position 86 for Tyrosine (K76T) has been found in almost all MDR field isolates. Studies conducted in the Greater Mekong subregion (Cambodia and Thailand) showed that increases in copy numbers of this gene are responsible for resistance to mefloquine and to increased risks for treatment failure with artesunate–mefloquine and artemether–lumefantrine (four-dose regimen only)

(Price *et al.* 2004 and Price *et al.* 2006). In *vitro* susceptibility to mefloquine, quinine, halofantrine and artemisinin increased when the *pfmdr1* copy numbers were reduced or when the parasites carried *pfmdr1* mutations (Sidhu *et al.* 2006 and Nkhoma *et al.* 2009). In South-East Asia, the presence of the Asn86Tyr mutation is a negative marker for gene amplification. Linkage disequilibrium between the Lys76Thr mutation on the *pfcrt* gene and the Asn86Tyr mutation on the *pfmdr1* gene has been observed in field studies. Highly chloroquine-resistant isolates appear to have at least the Lys76Thr and Ala220Ser mutations in the *pfcrt* gene and are generally associated with the Asn86Tyr mutation in the *pfmdr1* gene. *pfmdr1* mutations probably do not confer resistance to chloroquine but have an important modulatory effect (Roepe *et al.* 2009). *Pfmdr1* amplication and deamplification are relatively frequent events related to the rapid evolution of mefloquine resistance when the drug is used as monotherapy. In several field studies, artemether–lumefantrine appeared to select for the wild-type *pfmdr1* Asn86 allele in recurrent infection, which could be a marker for reduced susceptibility to lumefantrine (Sisowath *et al.* 2005, Dokomajilar *et al.* 2006, and Happi *et al.* 2009). Figure 2.8 shows the predicted structure and representative haplotypes of *P. falciparum* multidtug resistant transporter.

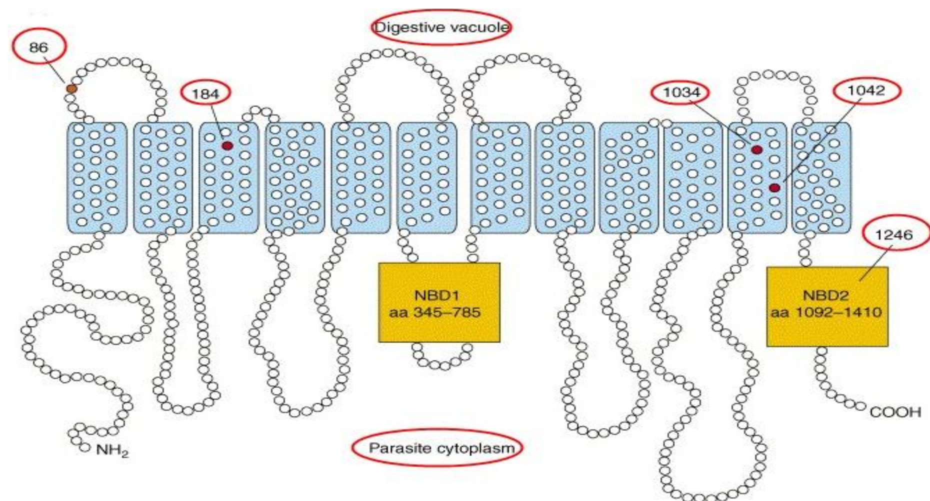

Figure 2.8. Predicted structure and representative haplotypes of *P. falciparum* multidtug resistant transporter. *Pfmdr1* is predicted to have 12 transmembrane domains, with its N and C termini located on the cytoplasmic side of the digestive vacuole membrane (Valderramos *et al.* 2006).

2.4.3 Analysis of resistance to common old antimalarial drugs

Since anti-malarial drug failure and resistance was observed in early 1960s, scientists around the globe have been relentlessly monitoring antimalarial drug efficacy and studying the mechanisms of underlying drug resistance. A number of genes involved or potentially involved in *P. falciparum* antimalarial drug resistance have been identified.

2.4.3.1 Early studies

Krogstad *et al.* (1987) investigated that chloroquine-resistant *P. falciparum* accumulate significantly less chloroquine than susceptible parasites. However, the reason for the lower accumulation of chloroquine was unknown. The resistant parasite has now been found to release chloroquine 40 to 50 times more rapidly than the susceptible parasite, although their initial rates of chloroquine accumulation are the same. These results suggest that a higher rate of chloroquine release explains the lower chloroquine accumulation, and thus the resistance observed in resistant *P. falciparum.*

Foote *et al.* (1990) found that chloroquine resistance (CQR) is biologically similar to the multiple drug resistance (MDR) phenotype of mammalian tumour cells, as both involve expulsion of drug from the cell and both can be reversed by calcium channel antagonists. A homologue (*pfmdr1*) of the mammalian multidrug resistance gene has been implicated in CQR because it is amplified in some CQR isolates of *P. falciparum* as is an *mdr* gene in MDR tumour cells. They showed that the complete sequences of *pfmdr1* genes from 2 CQ sensitive (CQS) *P. falciparum* isolates are identical. In 5 CQR isolates, 1-4 key nucleotide differences resulted in amino acid substitutions. On the basis of these substitutions, they have correctly predicted the CQS/CQR status of a further 34 out of 36 isolates. This is a paradox as CQR arises much less frequently than would be predicted if single point mutations were sufficient.

Fidock *et al.*, (2000) found that in a *P. falciparum* genetic cross maps to a 36 kb segment of chromosome 7 which segment harbors a 13-exon gene, *pfcrt*, having point

mutations that associate completely with CQR in parasite lines from Asia, Africa, and South America.

Djimde *et al.* (2001) measured the prevalence of *pfcrt* T76 and the prevalence of clinical chloroquine resistance and therapeutic failure at sentinel sites and calculated age adjusted genotype-resistance indices (GRIs) and genotype failure indices (GFIs). They found stable GRIs and GFIs at different sites in Mali, West Africa. This model permits mapping of chloroquine resistance using molecular tools in rapid and simple cross-sectional surveys.

In *P. falciparum*, chloroquine resistance is linked to multiple mutations in *Pfcrt*, a protein that likely functions as a transporter in the parasite's digestive vacuole membrane. Wellems and Plowe (2001) review recent field studies that support the central role of *Pfcrt* mutations in chloroquine resistance. These studies suggest chloroquine resistance arose in 4 distinct geographic foci and substantiate an important role of immunity in the outcomes of resistant infections after chloroquine treatment.

In 2001 Babiker *et al.* examined Polymorphisms in two *P. falciparum* genes, as were chloroquine responses of clones and isolates from a village in eastern Sudan. There was a significant association between an allele of the *P. falciparum* chloroquine resistance transporter gene (*pfcrt*-T76) and both *in vitro* and *in vivo* resistance. There was a less significant association with the multidrug resistance gene *pfmdr1*-Y86 allele. A significant association between *pfmdr1*- Y86 and *pfcrt*-T76 was apparent among resistant isolates, which suggests a joint action of the 2 genes in high-level chloroquine resistance.

Dorsey *et. al.* (2001) observed that the *pfcrt* lysinerthreonine mutation at position 76, which recently correlated fully with chloroquine resistance *in vitro*, was present in 100% of 114 isolates, of which about half were from patients who recovered clinically after chloroquine therapy. These results suggest that, although key *pfcrt* polymorphisms may be necessary for the elaboration of resistance to chloroquine in

areas with high levels of chloroquine resistance, other factors, such as host immunity, may contribute to clinical outcomes.

Cortese *et al.* (2002) studied haplotyping and microsatellite analysis of malaria from 5 regions of the South American Amazon and supported the conclusion that parasite harboring *pfdhps* mutation are found to share *pfcrt* mutation and CQ drug resistance.

2.4.3.2 Further probes into resistance

Djimde *et al. (*2003) investigated the clearance of malaria parasites carrying the key chloroquine resistance-conferring *Pfcrt* mutation K76T in patients treated with chloroquine. They found that the ability to clear these resistant parasites is strongly dependent on age (the best surrogate for protective immunity in endemic areas) suggesting that host immunity plays a critical role in the clearance of resistant *P. falciparum* infections.

Chloroquine resistance in *P. falciparum* is primarily conferred by mutations in *Pfcrt*. Parasites resistant to chloroquine can display hypersensitivity to other antimalarials; however, the patterns of cross- resistance are complex, and the genetic basis has remained elusive. Johnson *et al.* (2004) showed that stepwise selection for resistance to amantadine or halofantrine produced previously unknown *pfcrt* mutations (including S163R), which were associated with a loss of verapamil reversible chloroquine resistance. This was accompanied by restoration of efficient chloroquine binding to hematin in these selected lines. This S163R mutation provides insight into a mechanism by which *Pfcrt* could gate the transport of protonated chloroquine through the digestive vacuole membrane. Evidence for the presence of this mutation in a Southeast Asian isolate supports the argument for a broad role for *Pfcrt* in determining levels of susceptibility to structurally diverse antimalarials.

Duraisingh and Cowman (2005) observed that a strong association between possession of the wildtype form of *Pfmdr1*, amplification of *Pfmdr1* and resistance to hydrophobic drugs such as the arylaminoalcohol mefloquine and the endoperoxide artemisinin derivatives in field isolates. The arylaminoalcohol and endoperoxide

drugs are structurally unrelated drugs and this resistance resembles true multidrug resistance.

Pfcrt, an integral membrane protein with 10 predicted transmembrane domains, is a member of the drug/metabolite transporter superfamily and is located on the membrane of the intra-erythrocytic parasite's digestive vacuole. Specific polymorphisms in *Pfcrt* are tightly correlated with chloroquine resistance. Transfection studies of Bray *et al.* (2005) have now proven that *p*-mutations confer verapamil reversible chloroquine resistance *in vitro* and reveal their important role in resistance to quinine. Available evidence is consistent with the view that *Pfcrt* functions as a transporter directly mediating the efflux of chloroquine from the digestive vacuole.

From clinical studies Sidhu *et al.* (2006) have identified an association between an increased risk of MFQ, MFQ-artesunate, and LUM-artemether treatment failures and *Pfmdr1* gene amplification. To directly address the contribution that *Pfmdr1* copy number makes to drug resistance, they genetically disrupted 1 of the 2 *Pfmdr1* copies in the drug-resistant FCB line, which resulted in reduced *pfmdr1* mRNA and protein expression. These knockdown clones manifested a 3-fold decrease in MFQ IC50 values, compared with that for the FCB line, verifying the role played by *pfmdr1* expression levels in mediating resistance to MFQ. These clones also showed increased susceptibility to LUM, halofantrine, quinine, and ART. No change was observed for chloroquine. These results highlight the importance of *pfmdr1* copy number in determining *P. falciparum* susceptibility to multiple agents currently being used to combat malaria caused by multidrug-resistant parasites.

Sanchez *et al.* (2007) described that CQ resistance is linked to a K76T mutation in *Pfcrt*, a membrane-located food vacuolar protein and member of the drug-metabolite transporter superfamily, but there is as yet no agreed mechanism of how mutated *Pfcrt* brings about CQ resistance. Current models suggest that mutated *Pfcrt* acts either as a channel or a transporter of CQ, enabling CQ to leave the digestive food vacuole of the parasite, in which the CQ accumulates.

Parasite has developed resistance to specific drugs through alterations in predicted parasite transporter molecules such as P-glycoprotein homologue 1 (Pgh1) and *P. falciparum* CRT (*pfcrt*). Alterations in the expression of Pgh1 have been associated with modified susceptibility to a range of unrelated drugs. Johnson *et al.* (2008) have shown that the anticonvulsant phenobarbitone (PB) can induce reduced susceptibility to chloroquine (CQ) in *P. falciparum* that is associated with the increased expression of the drug transporter Pgh1 but not *Pfcrt*. Furthermore, they have investigated the proximal promoter regions from both *pfmdr1* and *pfcrt* and identified a number of putative binding sites for nuclear receptors with sequence similarities to regions known to be activated by PB in mammals. Whole-genome analysis has revealed a putative nuclear receptor gene, providing the first evidence that nuclear receptor-mediated responses to drug exposure may be a mechanism of gene regulation in *P. falciparum*.

Summers *et al.* (2012) concluded that mutations in the parasite's 'chloroquine resistance transporter' (*pfcrt*) are the primary cause of CQ resistance. Furthermore, changes in *pfcrt* (and in several other transport proteins) are associated with decreases or increases in the parasite's susceptibility to a number of other antimalarial drugs.

To identify novel mutant *pfcrt* genotypes and to reveal the genetic relatedness of *pfcrt* genotypes, a large-scale survey over a wide geographic area was performed by Takahashi *et al.* (2012). To date, at least 15 *pfcrt* genotypes, which are represented by combinations of five amino acids at positions 72-76, have been described in field isolates from various endemic regions.

The mutations of *Pfcrt* impart upon the ability to efflux chloroquine from the intracellular digestive vacuole, the site of drug action. Recent studies of Ecker *et al.* (2012) reveal that *Pfcrt* variants can also affect parasite fitness, protect immature gametocytes against chloroquine action, and alter *P. falciparum* susceptibility to current first-line therapies. These results highlight the need to be vigilant in screening for the appearance of novel *pfcrt* alleles that could contribute to new multidrug resistance phenotypes.

Pulcini *et al.* (2015) described *P. falciparum* lines subjected to selection by amantadine or blasticidin that carry *Pfcrt* mutations (C101F or L272F), causing the development of enlarged food vacuoles. These parasites also have increased sensitivity to chloroquine and some other quinoline antimalarials. A transgenic parasite line expressing the L272F variant of *Pfcrt* confirmed this increased chloroquine sensitivity and enlarged food vacuole phenotype. Furthermore, the introduction of the C101F or L272F mutation into a chloroquine-resistant variant of *Pfcrt* reduced the ability of this protein to transport chloroquine by approximately 93 and 82%, respectively, when expressed in Xenopus oocytes. These data provide, at least in part, a mechanistic explanation for the increased sensitivity of the mutant parasite lines to chloroquine. Taken together, these findings provide new insights into *Pfcrt* function and *Pfcrt*-mediated drug resistance, as well as the food vacuole, which is an important target of many antimalarial drugs.

2.4.3.3 Studies in Africa

To estimate the prevalence of the *pfcrt* K76T, *pfmdr1* N86Y, and *pfmdr1* D1246Y polymorphisms, isolates of *P. falciparum* from Senegal, West Africa, were analyzed by Thomas *et al.* (2002), and the results were compared to *in vitro* chloroquine susceptibility. By the *in vitro* DELI test, 31% of these samples were resistant to chloroquine. Polymerase chain reaction based assays and confirmatory sequencing demonstrated the *pfcrt* T76, *pfmdr1* Y86, and *pfmdr1* Y1246 alleles in 79%, 31%, and 2% of the isolates, respectively. All three mutant alleles were present in both *in vitro* susceptible and resistant isolates. On the basis of these findings, it appears that these molecular markers are not consistently predictive of *in vitro* chloroquine resistance in Senegal.

Malawi was the first African country to replace chloroquine with sulfadoxine pyrimethamine in 1993 in response to high rates of chloroquine-resistant falciparum malaria. To determine whether withdrawal of chloroquine had led to the re-emergence of chloroquine sensitivity, the prevalence of the *pfcrt* 76T molecular marker for chloroquine resistant *P. falciparum* malaria was retrospectively measured by Kublin

et al. (2003) in Blantyre, Malawi. The prevalence of the chloroquine-resistant *pfcrt* genotype decreased from 85% in 1992 to 13% in 2000. In 2001, chloroquine cleared 100% of 63 asymptomatic *P. falciparum* infections, no isolates were resistant to chloroquine *in vitro*, and no infections with the chloroquine-resistant *Pfcrt* genotype were detected. A concerted national effort to withdraw chloroquine from use has been followed by a return of chloroquine-sensitive falciparum malaria in Malawi. The researchers recommended that, reintroduction of chloroquine, ideally in combination with another antimalarial drug, should be considered in areas where chloroquine resistance has declined and safe and affordable alternatives remain unavailable.

Tinto *et al.* (2003) explored the relationship between *pfcrt* T76 and *Pfmdr1* Y86 mutations in *P. falciparum*, in samples collected from patients with uncomplicated malaria and tested *in vitro* and *in vivo* with chloroquine (CQ) in Burkina Faso which indicates that the two mutations ere strongly related. The *pfcrt* T76 mutation was found in 82% of the samples having the *pfmdr1* Y86 mutation too. However, only half of samples with *pfcrt* T76 mutation had also the *Pfmdr1* Y86 mutation. The latter was apparently associated with *in vitro* resistance but such association disappeared after adjusting for the presence of the *pfcrt* T76 mutation. This suggests that the occurrence of the *pfmdr1* Y86 mutation is dependent on that of *pfcrt* T76 mutation and could explain previous reports linking the *pfmdr1* Y86 mutation with CQ resistance (CQR). The isolates carrying both the *pfcrt* K76 and *pfmdr1* N86 alleles (wild/wild (WW)) and the single mutant *pfmdr1* Y86 (WM) had the lowest IC50 geometric mean (GMIC50) values, while those carrying both *pfcrt* T76/*pfmdr1* Y86 alleles (mutant/mutant (MM)), and the single mutant *pfcrt* T76 (MW) had the highest.

Ochong *et al.* (2003) report an association of the *P. falciparum* chloroquine resistance transporter (*Pfcrt*) gene and the *P. falciparum* multiple drug resistance 1 (*Pfmdr1*) gene, two chloroquine resistance markers, with chloroquine and amodiaquine efficacy *in vivo* in southern Sudan. The data show that the allele of the *Pfcrt* gene with a lysine o threonine change at codon 76 is strongly associated with both chloroquine and amodiaquine resistance. No such association was observed with the *Pfmdr1* gene.

In Africa, Sisowath *et al.* (2005) have performed an artemether-lumefantrine (Coartem; Novartis) follow-up clinical trial in Zanzibar, in which *Pfcrt* K76T and *Pfmdr1* N86Y frequencies were determined before drug administration and in all recurrent parasites during a follow-up period of 42 days. A significant increase in *Pfmdr1* 86N was observed after exposure to the drug. It points to 86N as a potential marker of lumefantrine resistance *in vivo*, while suggesting that Coartem is not robust enough to avoid selection of resistance-associated mutations in some malarial settings.

With an aim to examine the effect of transient removal of drug pressure, Ord *et al.* (2007) analyzed seasonal changes in the prevalence of chloroquine (CQ)–resistant parasite genotypes in Gambia. Parasite isolates from 441 children presenting with uncomplicated falciparum malaria over 5 seasons (1998 –2002) were linked to weekly rainfall data. The prevalence of CQ-resistant parasites increased slightly over 5 years, with the 76T allele of *Pfcrt* and the 86Y allele of *Pfmdr1* becoming significantly more common. However, intra-seasonal analysis showed that these alleles decreased in prevalence each dry season. Wild-type parasites with respect to both loci predominated as transmission began each year, with resistant parasite becoming more common as drug use increased. This pattern was seen for both *pfcrt*-76T and *pfmdr1*-86Y and could not be explained by seasonal changes in the clonal complexity of infections.

Between April and June 2010, 313 children below 10 years of age living in Brazzaville, Congo were screened for *P. falciparum* infection using microscopy and polymerase chain reaction (PCR). The *pfcrt* K76T mutation was detected using nested PCR followed by restriction endonuclease digestion. The prevalence of the mutant *pfcrt* allele (T76) in the isolates was 92% (Koukouikila-Koussounda *et al.* 2012).

Gbotosho *et al.* (2012) analyzed the effect of antimalarial drug selection on *pfcrt* and *pfmdr1* polymorphisms in *P. falciparum* isolates from two distinct geographical locations from Nigeria and Brazil, using nested polymerase chain reaction and direct DNA sequencing approaches. All isolates from Brazil and 72% from Nigeria harbored

the mutant SVMNT and CVIET *pfcrt* haplotype, respectively. The *pfcrt* CVMNT haplotype was also observed in (7%) of the Nigerian samples. One hundred percent (100%) and 54% of the parasites from Brazil and Nigeria, respectively, harbored wild-type *pfmdr1*Asn86. They provide first evidence of emergence of the CVMNT haplotype in West Africa.

West African country of Ghana introduced artemisinin-based combination therapy in 2005. Duah *et al.* (2013) investigated the *P. falciparum* multidrug resistance gene (*pfmdr1*) copy number, mutations and the chloroquine resistance transporter gene (*pfcrt*) mutations. Increased *pfmdr1* gene copy number was observed in the isolates analysed and this finding has implications for the use of ACT in the country although no resistance has been reported. The decreasing trend in the prevalence of chloroquine resistance markers after change of treatment policy presents the possibility for future introduction of chloroquine as prophylaxis for malaria risk groups such as children and pregnant women in Ghana.

Chloroquine (CQ) use in Mozambique was stopped in 2002 and artemether lumefantrine (AL) was implemented in 2008. In light of no use of CQ and extensive use of AL, Thomsen *et al.* (2013) determined the frequency of molecular markers of *P. falciparum* drug resistance/tolerance to CQ and AL in persons living in two coastal villages situated 500 km north of Maputo.. The *P. falciparum* chloroquine resistance transporter gene CVMNK wild type increased in frequency from 43.9% in 2009 to 66.4% in 2010, and combined *P. falciparum* multidrug resistance gene 1 N86-184F-D1246 haplotype increased significantly between years. The combination of P.falciparum chloroquine resistance transporter gene CVMNK and P.falciparum multidrug resistance gene NFD increased from 24.3% (2009) to 45.3% in (2010, P = 0.017). The rapid changes observed may largely be caused by decreased use of CQ and large-scale use of AL.

Nwakanma *et al.* (2014) analyzed genome-wide SNPs in *P. falciparum* drug resistance genes of a Gambian Population between 1984 and 2008. They found that

resistance alleles increased from very low frequencies, peaking in 2000 for chloroquine resistance associated crt and mdr1 genes.

Six years after withdrawal of chloroquine and introduction of artemisinin-based combinations in West African country of Niger, Salissou *et al.* (2014) observed low prevalence of *pfcrt* resistance alleles among patients with uncomplicated falciparum malaria. *pfcrt* haplotypes (aa 56–118) and *ex vivo* responses to CQ and amodiaquine were characterized for 26 isolates collected in South Niger from children under 15 years of age suffering from uncomplicated *falciparum* malaria. The wild-type *Pfcrt* haplotype CVMNK was found in 22 of the 26 isolates, with CVIET sequences observed in only three of the samples. Findings of the study suggested a significant decline in CQ-resistant genotypes since the previous estimates for Niger were obtained.

In central African country of Cameroon Moyeh *et al.* (2018) conducted a study to assess the effect of antimalarial therapy changes on the prevalence of molecular markers of resistance from 2003 to 2013. The prevalence of individual marker polymorphisms and haplotypes was compared in two study periods namely 2003-2005 and 2009-2013. Alleles conferring resistance to 4-aminoquinolines in the *Pfcrt* 76T and *Pfmdr1* 86Y, 184F, and 1246Y genotypes showed a significant reduction of 97.0% to 66.9%, 83.6% to 45.2%, 97.3% to 56.0%, and 3.1% to 0.0%, respectively. No difference was observed in SNPs associated with antifolate drugs resistance 51I, 59R, 108N, or 540E. Haplotype analysis in the *Pfmdr1* gene showed a reduction in the YFD from 75.90% to 42.2%, and an increase in the NYD (2.9% to 30.1%;). The results indicated a gradual return of the 4-aminoquinoline sensitive genotype while the antifolate resistant genotypes increased to saturation.

Yao *et al.* (2018) investigated the gene mutations of *P. falciparum* using blood samples from the Chinese migrant workers returned from Africa and Southeast Asia (SEA) between 2011 and 2016, in order to identify molecular markers associated with drug resistance. After analyzing polymorphisms in *pfcrt*, *pfmdr1*, and k13-propeller genes and the haplotype patterns of *Pfcrt* and *Pfmdr1* they showed the presence of

four haplotypes of *Pfcrt* codons 72-76, including CVMNK (wild type), S VMN T, and CV IET (mutation type), CV M /I N /E K /T (mixed type), with 50.57%, 1.14%, 25.00%, and 23.30% prevalence, respectively. For *Pfmdr1*, N86 Y (22.28%) and Y184 F (60.01%) were the main prevalent mutations. The prevalence of mutation at position 550, 561, 575, and 589 of K13-propeller were 1.09%, 0.54%, 0.54% and 0.54%, respectively. These data suggested that *Pfcrt*, *Pfmdr1*, and K13-propeller polymorphisms are the potential markers to assess drug resistance of *P. falciparum* in China, Africa and SEA.

Xu *et al.* (2018) analyzed polymorphisms of the*Pfcrt*, *Pfmdr1*, and *Pfkelch13* genes in 282 *P. falciparum* cases returned from Africa to Shandong province of China between 2012 and 2015. Among the isolates, polymorphisms were detected in codons 74–76 of *Pfcrt* and 86, 184, 1246 of *Pfmdr1*, among which K76T (36.6%) and Y184F (60.7%) were the most prevalent, respectively. Six *Pfcrt* haplotypes and 11 *Pfmdr1* haplotypes were identified.

Ocan *et al.* (2019[a]) reviewed published articles to establish prevalence of *Pfcrt* 76T and *Pfmdr1* 86Y alleles in malaria affected countries following official discontinuation of chloroquine use. Prevalence of *Pfcrt* 76T was as follows; East Africa 48.9% (2528/5242), Southern Africa 18.6% (373/2163), West Africa 58.3% (3321/6608), Asia 80.2% (1951/2436). Prevalence of *Pfmdr1* 86Y was; East Africa 32.4% (1447/5722), Southern Africa 36.1% (544/1640), West Africa 52.2% (1986/4200), Asia 46.4% (1276/2217). Over half, 52.6% (20/38) of included studies reported continued unofficial chloroquine use following policy change. The prevalence of chloroquine resistance alleles among *P. falciparum* parasites have steadily declined since discontinuation of chloroquine use. However, *Pfcrt* K76T and *Pfmdr1* N86Y mutations still persist at moderate frequencies in most malaria affected countries.

2.4.3.4 Studies in Asia

Rastaghi *et al.* (2008) investigated a study within 64 samples from patients with uncomplicated falciparum malaria from Sarbaz district in southeast of Iran, the prevalence of K76T mutations in *pfcrt* gene were investigated by *in vivo* and nested-PCR followed restriction enzyme digestion methods. The occurrence of the K76T mutation was very high (93.75%) among these filed isolates. Only 4 of 64 isolates harbored wild type K76 codon and no case was a mixed of K76 and 76T codons. All of the 22 (100%) chloroquine-resistant and 16.7% of sensitive isolates were found to harbor the 76T mutation and none was found to contain the wild type (K76) allele.

Atroosh *et al.* (2012) revealed significant associations of *pfcrt* K76T, *pfcrt* N326S and *pfcrt* I356T mutations with parasitaemia in a study conducted in Malaysia to find out the magnitude of *P. falciparum* CQ resistance.

In Iran, 4 years after implementation of artemisinin combination therapy Pirahmadi *et al.* (2013) conducted a mutation analysis in *pfmdr1* and pfmrp1 as potential candidate genes for artemisinin resistance in *P. falciparum* clinical isolates and suggested that, multidrug resistance 1 (*pfmdr1*) (N86Y/Y184F/S1034C/N1042D/F1226Y/D1246Y) and multidrug resistance protein 1 (pfmrp1) (H191Y/S437A/I876V/F1390I/K1466R) genes that are probably associated with artemisinin as well as chloroquine resistance transporter (*pfcrt*) 76T in *P. falciparum* clinical isolates. The copy number of *pfmdr1* gene was screened for its association with *pfmdr1* mutations to incriminate artemisinin resistance by using nested PCR-RFLP and sequencing analysis. In total, 42.5% of the examined isolates carried both *pfmdr1* 86Y and *pfcrt* 76T and none of the parasites simultaneously harbored *pfcrt* 76T, *pfmdr1* 86Y, 184F and pfmrp1 191Y, 437A, 876V, 1390I mutations. In addition, the copy number of *pfmdr1* gene (N = 1) was similar as a sensitive isolate, 3D7, to artemisinin.

2.4.3.5 Studies in South East Asian region

Sequence analysis of polymorphisms in *pfcrt* (position 76) and *pfmdr1* (positions 86, 184, 1034, 1042, and 1246) and measurement of *in vitro* drug sensitivities were done by Pickard *et al.* (2003) in 65 *P. falciparum* isolates from Thailand, Myanmar, Vietnam, and Bangladesh. The *pfcrt* Thr76 polymorphism was present in 97% of samples, consistent with observations that chloroquine resistance is well established in this region. Polymorphisms in *pfmdr1* showed that mefloquine resistance with sensitivity and specificity of 94 and 91% respectively.

Ngo *et al.* (2003) have analyzed artemisinin sensitivity in *P. falciparum* isolates obtained from patients in South Vietnam and show that artemisinin sensitivity does not differ before and after drug treatment. There was an increase in the level of mefloquine resistance in the isolates after drug treatment that was concomitant with a decrease in chloroquine resistance, suggesting that treatment with artemisinin has selected for increased mefloquine resistance. Mutations in the *Pfmdr1* gene, previously shown to be associated with sensitivity to mefloquine, were selected against. All isolates resistant to chloroquine encoded Thr-76 in the *Pfcrt* gene consistent with an essential role in the mechanism of chloroquine resistance. Mutations in *Pfmdr1* also were linked to chloroquine resistance. High levels of mutation in *dhfr* and *dhps* genes, which have previously been associated with Fansidar resistance, also were found, suggesting that this drug would not be useful for malaria control in this part of Vietnam.

Anderson *et al.* (2005) examined polymorphisms in *Pfcrt* and *Pfmdr1* genes and *in vitro* resistance to eight drugs in parasites collected from the Thailand-Burma border. They first examined associations between genotype and drug response in 108 unique single-clone parasite isolates and found strong associations between single nucleotide polymorphisms in Pfmdr and mefloquine (MFQ), artesunate (AS), and lumefantrine (LUM) response. Furthermore, they found no correlation between chloroquine and quinine responses although they did find expected strong correlations between MFQ, QN, AS, DHA, and LUM.

In an endemic area along the Indo-Bangladesh border, Goswami *et al.* (2014) studied the frequency distribution of *pfcrt* K76T and *pfmdr1* N86Y mutations and their association with chloroquine susceptibility. Chloroquine resistance in *P. falciparum* was found to be associated with mutations in *pfcrt* and *pfmdr1* genes. Out of 63 cases, 44 (69.8%) responded adequately to chloroquine treatment. *Pfcrt* K76T mutation was recorded in 100% of the treatment failure cases, whereas *pfmdr1* N86Y mutation was found in 52.6% of the cases only.

In a four years' monitoring study of *in vitro* sensitivity and candidate molecular markers of resistance of *P. falciparum* to artesunate - mefloquine combination in the Thai-Myanmar border Phompradit *et al.* (2014) observed high prevalence of CQ and MQ-resistant *P. falciparum* isolates during the four-year observation period (2006–2009) along the areas of Thai-Cambodian and Thai-Myanmar borders. AS drug sensitivity declined while QN sensitivity improved. *Pfmdr1* and pfmrp1 appear to be the key genes that modulate multidrug resistance in *P. falciparum.*

Patel *et al.* (2017) analyzed mutations in six different genes of *P. falciparum* (*crt*, *mdr1*, *dhfr*, *dhps, ATPase6* and *K-13* propeller) that confer resistance to chloroquine, sulphadoxine-pyrimethamine and artemisinin-based combination therapy, in samples from Chhattisgarh India. Seventy-eight percent of the samples were found to have a *pfcrt* mutation (53% double, 24% triple and 1% single mutant), and 59% of *pfmdr1* genes were found to have an N86Y mutation. Double mutations were recorded in *pfdhfr* gene among 76% of the samples while only 6% of the samples harbored mutant genotypes in *pfdhps*. The high level of *pfcrt* mutations suggests that parasite resistance to chloroquine is almost at a fixed level, whereas resistance to SP is evolving in the population and parasites remain sensitive to artemisinin derivatives.

Ross *et al.* (2018) carried out genomic analysis of Cambodian isolates collected between 2011 and 2012 and revealed a rapid increase in the prevalence of novel mutations in the *P. falciparum* chloroquine resistance transporter *pfcrt* following DHA + PPQ implementation. These mutations occur in parasites harboring the K13

C580Y artemisinin resistance marker. Findings of the research provide compelling evidence that emerging mutations in *Pfcrt* can serve as a molecular marker and mediator of PPQ resistance.

A study focusing screening of *pfmdr1* polymorphism was conducted in 2015 by Goomber *et al.* (2018) in three Indian state Mizoram, Tripura, Meghalaya which have borders with Bangladesh and Myanmar. They observed marked predominance of Plasmodium isolates with *Pfmdr1* wild type alleles for all codons under study i.e. 86, 184, 1246. Spatially, Plasmodium isolates from Mizoram were most diverse with co-existence of genotype with NYD, YYD, NFD haplotypes, followed by Tripura. Isolates from Meghalaya were of all NYD haplotype. Temporal study showed distinct rise in proportion of *Pfmdr1* wild type N86 allele since introduction of Artemether-Lumefantrine as first line antimalarial.

2.4.3.6 Studies in Bangladesh

Forty-four *P. falciparum* isolates from Bangladesh and 22 from western Thailand were successfully tested by Noedl *et al.* (2003) for their drug susceptibility. High degrees of resistance were observed against chloroquine with geometric mean IC 50s of 114.25 and 120.5 nM, respectively, for Bangladesh and western Thailand. Most isolates from both sites were sensitive to quinine, and all were sensitive to artesunate. Many isolates were considered *in vitro* resistant to mefloquine, but the geometric mean IC50 for the Thai isolates (98.79 nM) was 1.6 times (*P* 0.002) higher than that of isolates from Bangladesh (60.3 nM). The high prevalence of *in vitro* mefloquine resistance in Bangladesh suggests that close surveillance is necessary to delay widespread multidrug resistant problems in the area.

Ingrid *et al.* (2004) genotyped 203 blood samples from Chittagong Hill Tracts of Bangladesh for parasite mutations related to chloroquine (*pfcrt* and *pfmdr1* genes) or SP resistance (*dhfr* and *dhps*). The majority of infections carried mutations associated with chloroquine resistance: 94% at *pfcrt* and 70% at *pfmdr1* Sp-resistant genotypes were also frequent: 99% and 73% of parasites carried two or more mutations at *dhfr*

and *dhps*, respectively. The frequency of alleles at *dhfr*, *dhps* and *pfmdr1* was similar in cases that were successfully treated and those that recrudesced. The genotyping results suggest that neither chloroquine nor SP can be considered a reliable treatment for *P. falciparum* malaria any longer in this area of Bangladesh.

In Bangladesh, despite the official introduction of artemisinin combination therapy in 2004, chloroquine+sulfadoxine/pyrimethamine continued to be used for the treatment of uncomplicated malaria. To assess the distribution of *pfcrt*, *pfmdr1*, *dhfr*, and *dhps* genotypes in *P. falciparum*, Marma *et al.* (2010) conducted hospital- and community-based surveys in Bandarban, Bangladesh in 2007 and 2008. Using nested PCR followed by digestion, they genotyped 139 *P. falciparum* isolates and found fixation of a mutation at position 76 in *pfcrt* and low prevalence of a mutation at position 86 in *pfmdr1*. In *dhfr*, the highest pyrimethamine resistant genotype quadruple mutant was found in 19% of isolates, which is significantly higher prevalence than reported in a previous study in Khagrachari (1%) in 2002. Microsatellite haplotypes flanking *dhfr* of the quadruple mutants in Bangladesh were identical or very similar to those found in Thailand and Cambodia, indicating a common origin for the mutant in these countries. These observations suggest that the higher prevalence of the *dhfr* quadruple mutant in Bandarban is because of parasite migration from Myanmar. However, continuous use of sulfadoxine/pyrimethamine would have also played a role through selection for the *dhfr* quadruple mutant.

Kawai *et al.* (2011) investigated the efficacy of chloroquine (CQ) plus primaquine (PQ) and the mutation status of the CQ resistance transporter (*pfcrt*) gene of infecting *P. falciparum* in 45 uncomplicated falciparum malaria patients in Rangamati, Bangladesh in 2004. The total failure rate was 57.8%. One or two *pfcrt* sequences (CIETH and SMNTH at positions 72, 74-76, and 97, mutation underlined with K76T mutation known to be related to CQ-resistant phenotype were detected in 38(84%) patients' blood samples. Of the 38 patients, in total 15 patients (14/25 minority-indigenous and 1/13 Bengali patients) resulted in adequate clinical and parasitological response (ACPR).

Akter *et al.* (2012) conducted an analysis of polymorphic and drug resistant genotype on 33 paired recrudescent infections after drug treatment in the period 2004 to 2008 in the Chittagong Hill Tracts Bangladesh. Overall the multiplicity of infection for MSP-1 was 2.7 with a slightly smaller parasite diversity post-treatment. The 13 monoclonal infections by both GLURP and MSP-1 were evenly divided between pre- and post-treatment. The MSP-1 MAD block was most frequent in 66 of the samples. The prevalence of the K76T *Pfcrt* chloroquine resistant allele was approximately 82% of the samples, while the resistant *Pfmdr1* N86Y was present in 33% of the samples. Interestingly, the post-treatment samples had a small but significantly higher frequency of the sensitive *Pfcrt* alleles by RT-PCR.

Huque *et al.* (2013) reviewed and summarized antimalarial drug resistance data from Bangladesh published until June 2013. Published studies indicate that *P. falciparum* shows varying levels of resistance to chloroquine, mefloquine and sulfadoxine-pyrimethamine. Combination therapy of chloroquine and primaquine has proven ineffective and combinations of sulfadoxine-pyrimethamine with either quinine or chloroquine have also shown poor efficacy. Recent studies indicate that artemisinin derivatives, such as artesunate, remain highly efficacious in treating *P. falciparum* malaria. Available data suggest that artemisinins, quinine, doxycyline, mefloquine–artesunate and azithromycin–artesunate combination therapy remain efficacious in the treatment of *P. falciparum* malaria in Bangladesh.

Alam *et al.* (2017) carried out molecular analysis of 130 *P. falciparum* isolates collected between August 2014 and January 2015 from Bandarban, an endemic district of Chittagong Hill Tracts of Bangladesh. In that study, *pfcrt* mutation K76T, associated with chloroquine resistance was found in 81.5% (106/130) of cases and the *pfmdr1* mutation N86Y in 13.9% (18/130) cases. No single nucleotide polymorphisms were observed in the *k13* propeller region.

2.4.4 *P. falciparum* resistance to artemisinin

Artemisinin (ART) derivatives have become the keystone of malaria treatment and control (WHO 2010[b]). ART has the advantage of killing all asexual blood stages of *P. falciparum* parasites, as well as affecting sexual development (White, 2008), resulting in rapid clinical and parasitological cure at an individual level, and a reduction in malaria transmission rates on a public health scale (WHO 2010[b]).

Artemisinin-based combination therapies (ACTs) are recommended by WHO as the first-and second-line treatment for uncomplicated *P. falciparum* malaria as well as for chloroquine-resistant *P. vivax* malaria. ACTs combine an artemisinin derivative with a partner drug. The role of the artemisinin compound is to reduce the number of parasites during the first three days of treatment (reduction of parasite biomass), while the role of the partner drug is to eliminate the remaining parasites (cure) (WHO 2018[d]).

Artemisinin resistance is defined as delayed parasite clearance following treatment with an artesunate monotherapy or with an artemisinin-based combination therapy. This represents partial resistance. However, delayed parasite clearance does not necessarily lead to treatment failure (WHO 2018[d]). The ART-R phenotype is recognized clinically as a prolongation of parasitemia clearance as measured by peripheral blood smears (delayed parasite clearance time; DPCT) in patients with uncomplicated falciparum malaria.

Historically, Southeast Asia has been the epicenter of malaria drug-resistance development. Resistance to all major antimalarials has emerged there. *P. falciparum* resistance to artemisinin was first reported from western Cambodia in 2008 (Dondrop *et al.* 2010) and has already spread across the Greater Mekong subregion (Menard *et al.* 2016). In the Greater Mekong Subregion (GMS), high treatment failure rates following treatment with an ACT have almost always been observed in areas where there is concomitant partial resistance to artemisinin and resistance to the ACT partner drug. Outside the GMS, treatment failure with ACTs (artesunate-amodiaquine and

artesunate-sulfadoxine-pyrimethamine) has occurred in the absence of artemisinin partial resistance mainly due to partner drug resistance. The independent emergence of artemisinin partial resistance in multiple locations in the GMS and the emergence of multidrug resistance are causing failure of ACT (Fig 2.9), that have led WHO to recommend the elimination of malaria in this region. (WHO 2018[e]).

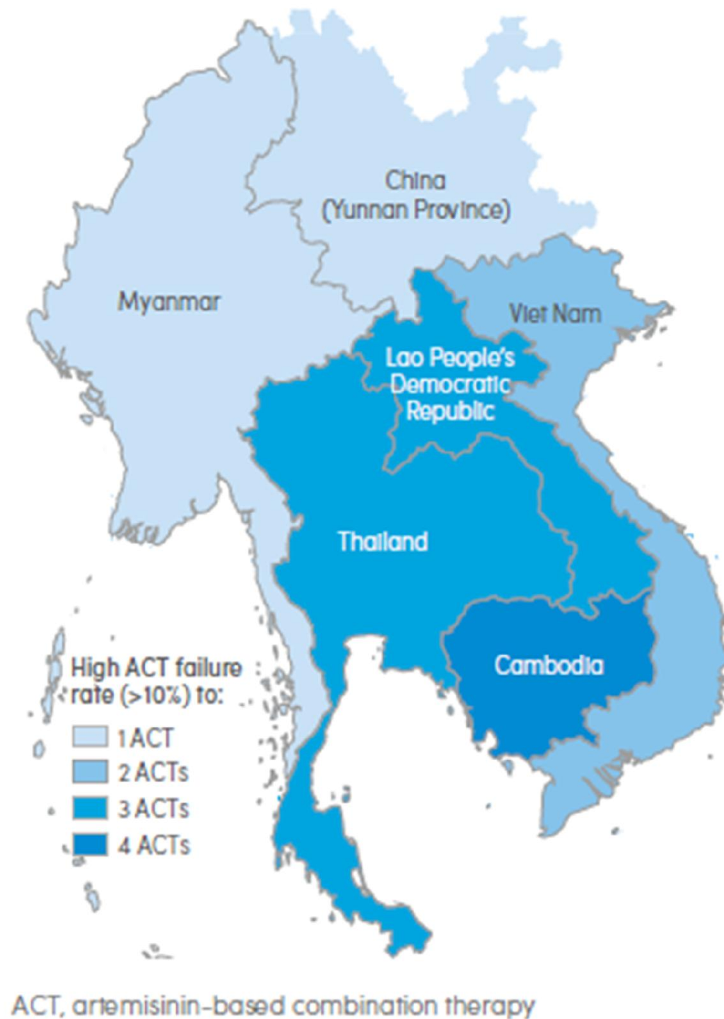

Figure 2.9. Numbers of ACTs failing in the Greater Mekong Subregion (WHO 2018[d]): There are currently five ACTs recommended by WHO: artemether-lumefantrine, artesunate-amodiaquine, artesunate-mefloquine, artesunate-sulfadoxine-pyrimethami ne and dihydroartemisinin-piperaquine. Blue-colour gradient shows failure of number of ACTs.

Hott *et al.* (2015) demonstrated that artemisinin-resistant *P. falciparum* has evolved a novel mechanism of phenotypic resistance to artemisinin drugs linked to abnormal cell cycle regulation. The resistant parasites exhibit altered patterns of development that result in reduced exposure to drug at the most susceptible stage of development in erythrocytes (trophozoites) and increased exposure in the most resistant stage (rings). In addition, a novel *in vitro* delayed clearance assay (DCA) that assesses drug effects on asexual stages was found to correlate with parasite clearance half-life *in vivo* as well as with mutations in the Kelch domain gene associated with resistance (Pf3D7_1343700). Importantly, all of the resistance phenotypes were stable in cloned parasites for more than 2 years without drug pressure.

2.4.4.1 Molecular marker of *P. falciparum* resistance to artemisinin

Global malaria control and elimination activities are threatened by development of resistance of *P. falciparum* to artemisinin derivatives in South-east Asia. To monitor the spread of artemisinin resistance, a molecular marker was urgently needed.

In 2014, Ariey *et al.* identified mutations in K 13 propeller gene of *P. falciparum* to be associated with artemisinin resistance. Using whole-genome sequencing of an artemisinin-resistant parasite line from Africa and clinical parasite isolates from Cambodia, they could associate mutations in the PF3D7_1343700 kelch propeller domain ('K13-propeller') with artemisinin resistance *in vitro* and *in vivo*. Strong correlations between the presence of a mutant allele, *in vitro* parasite survival rates and *in vivo* parasite clearance rates indicated that K13-propeller mutations are important determinants of artemisinin resistance. K13-propeller polymorphism constitutes a useful molecular marker for large-scale surveillance efforts (Ariey *et al.* 2014). The discovery,that took place less than 10 years after the detection of the first clinical cases of artemisinin resistance in western Cambodia has been validated by several studies in the Mekong region (Menard *et al.* 2015).

Not all of the non-synonymous propeller-region K13 mutants reported indicate the emergence of artemisinin resistance; rather, such mutants can represent passer-by

genotypes in the absence of evidence for the selection of the mutant K13 genotype. In addition, different K13 mutations have varying effects on the clearance phenotype.

The validation of a K13 mutation as a marker for artemisinin resistance requires that the mutation has been correlated with slow clearance in clinical studies and also correlated with reduced *in vitro* drug sensitivity (e.g., ring-stage assay ‑ RSA0-3h) using fresh isolates, or reduced *in vitro* sensitivity resulting from the insertion of the K13 mutant in transfection studies. If a K13 mutation has only been shown to be correlated with delayed parasite clearance during clinical trials but not validated by *in vitro* data, it is labelled a candidate/associated marker. (WHO 2018[d]).

In 2014 Menard *et al.* (2016) launched the K13 Artemisinin Resistance Multicenter Assessment (KARMA) study with an aim to assess the global distribution of K13 polymorphisms. They analyzed parasites that were collected from regions in which malaria is endemic, using a dedicated molecular toolbox and validation procedures for sequence data. They identified 108 nonsynonymous K13 mutations, which showed marked geographic disparity in their frequency and distribution. In Asia, 36.5% of the K13 mutations were distributed within two areas — one in Cambodia, Vietnam, and Laos and the other in western Thailand, Myanmar, and China — with no overlap. The study found no evidence of artemisinin resistance outside Southeast Asia and China, where resistance-associated K13 mutations were confined. In Africa, they observed a broad array of rare nonsynonymous mutations that were not associated with delayed parasite clearance. The common African A578S allele was not associated with clinical or *in vitro* resistance to artemisinin.

To date, around 200 non-synonymous mutations in the K13 gene have been reported. Figure 2.2 presents a current list of markers either validated, or candidates/associated with artemisinin resistance identified through correlation with delayed parasite clearance in clinical studies and/or *in vitro* drug sensitivity assays (e.g., ring-stage assay –RSA0-3h).

Table 2.2. List of markers either validated or candidates/associated with artemisinin resistance (WHO 2018[d])

Validated		Associated/ Candidates	
F446I	P553L	P441L	G538V
N458Y	R561H	G449A	V568G
M476I	C580Y	C469F	P574L
Y493H		A481V	F673I
R539T		P527H	A675V
I543T		N537I	

2.4.4.2 Studies in GMS

Chotivanich *et al.* (2014) demonstrated that slow parasite clearance in falciparum malaria in western Cambodia resulted from reduced ring-stage susceptibility. In Pailin, western Cambodia, where artemisinin-resistant *P. falciparum* is prevalent, the TMI test mean (95% confidence interval) 50% inhibitory concentration (IC50) for artesunate was 6.8 (5.2 to 8.3) ng/ml compared with 1.5 (1.2 to 1.8) ng/ml for the standard 48-hWHO test (*P*_0.001). TMI IC50s correlated significantly with the *in vivo* responses to artesunate (parasite clearance time [*r*_0.44, *P*_0.001] and parasite clearance half-life [*r*_0.46, *P*_0.001]), whereas the standard 48-h test values did not. On continuous culture of two resistant isolates, the artemisinin-resistant phenotype was lost after 6 weeks (IC50s fell from 10 and 12 ng/ml to 2.7 and 3 ng/ml, respectively).

A molecular epidemiological observation was conducted by Imwong *et al.* (2017) on artemisinin-resistant malaria between Jan 1, 2008, and Dec 31, 2015, they collected 434 isolates. In 2014–15, a single long *pfk13* C580Y haplotype (–50 to +31·5 kb) lineage, which emerged in western Cambodia in 2008, was detected in 65 of 88 isolates from northeastern Thailand, 86 of 111 isolates from southern Laos, and 14 of 14 isolates from western Cambodia, signifying a hard transnational selective sweep. *Pfplasmepsin2* amplification occurred only within this lineage, and by 2015 these

closely related parasites were found in ten of the 14 isolates from Cambodia and 15 of 15 isolates from northeastern Thailand. C580Y mutated parasites from Myanmar had a different genetic origin. The results suggested that the dominant artemisinin-resistant *P falciparum* C580Y lineage probably arose in western Cambodia and then spread to Thailand and Laos, outcompeting other parasites and acquiring piperaquine resistance. The emergence and spread of fit artemisinin-resistant *P falciparum* parasite lineages, which then acquire partner drug resistance across the Greater Mekong subregion, threatens regional malaria control and elimination goals.

Woodrow *et al.* (2017) reviewed clinical efficacy data originated between 2000 and 2015 from South East Asian region that provides strong evidence that the loss of first-line ACTs in western Cambodia, first artesunate mefloquine and then DHA-piperaquine, can be attributed primarily to K13 mutated parasites. The ring-stage activity of artemisinins is therefore critical for the sustained efficacy of ACTs; once it is lost, rapid selection of partner drug resistance and ACT failure are inevitable consequences. Consensus methods for monitoring artemisinin resistance are now available. Despite increased investment in regional control activities, ACTs are failing across an expanding area of the Greater Mekong subregion. Although multiple K13 mutations have arisen independently, successful multidrug-resistant parasite genotypes are taking over and threaten to spread to India and Africa. Stronger containment efforts and new approaches to sustaining long-term efficacy of antimalarial regimens are needed to prevent a global malaria emergency.

Distinct alleles originating from multiple independent events of emergence have been observed in South-East Asia. A worldwide surveillance of parasite genotypes, in has yielded evidence of two distinct epidemiological regions in the GMS: western GMS consisting of China (Yunnan province), Myanmar and western Thailand bordering Myanmar; and eastern GMS consisting of Cambodia, Lao PDR, Viet Nam and eastern Thailand bordering Cambodia and Lao PDR. (Menard *et al.* 2016). Five different mutants have been found to have the highest prevalence: F446I, R539T, I543T, P574L and C580Y. Certain mutations have only been found in a particular region. For example, the I543T mutation has only been detected in eastern GMS, whereas F446I

has only been detected in western GMS. The F446I mutant appears to be associated with an intermediate rate of delayed clearances (WHO 2018[d]) (Figure 2.10).

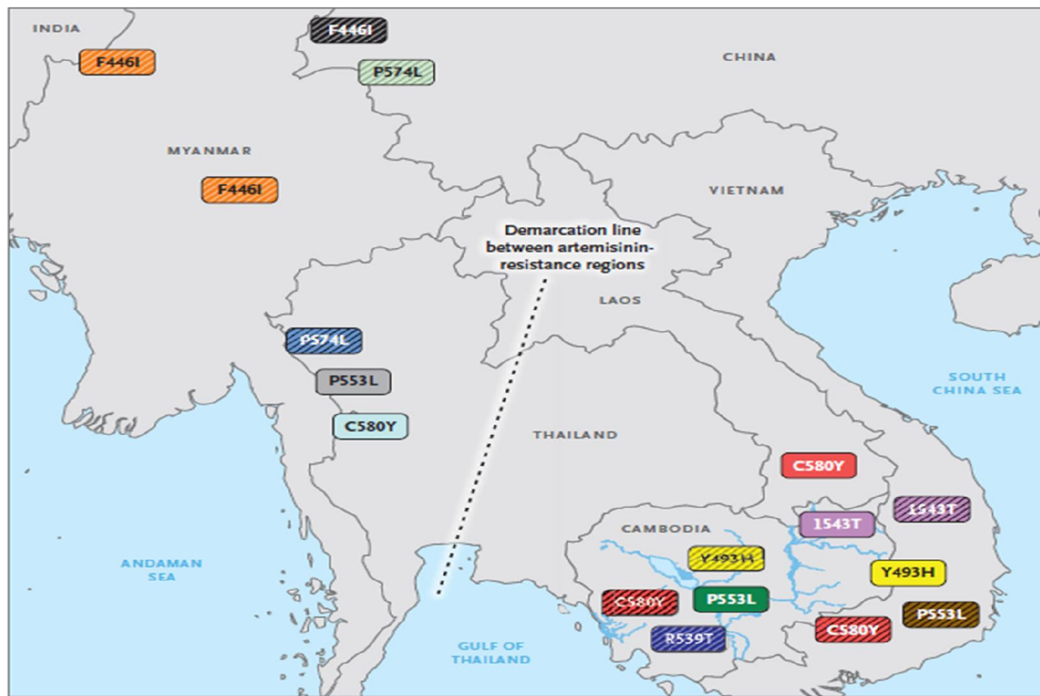

Figure 2.10. Overview of the distribution of the flanking haplotypes of the c580y, y493h, r539t, i543t, p553l, p574l, and f446i nonsynonymous mutations in k13 in two regions in asia (Menard *et al.* 2016)

The C580Y mutation has been found in several genetic backgrounds (haplotypes) throughout the GMS. The frequencies of different K13 C580Y haplotypes vary by region. The prevalence of one specific K13 C580Y haplotype has been increasing and replacing other haplotypes in eastern GMS, which indicates a selective sweep in this part of the GMS. The C580Y mutation appears to have reached fixation in areas of Cambodia where almost all resistant parasites are found to have this specific K13 mutation. The C580Y mutation has been found at a prevalence of up to 70% at the border between Thailand and Myanmar. Studies have shown that the predominant K13 mutants found in Myanmar do not appear to have spread from Cambodia, but likely arose independently. K13 mutations remain rare or unrelated to partial artemisinin resistance in Bangladesh and northeast India (WHO 2018[d]) (Figure 2.11).

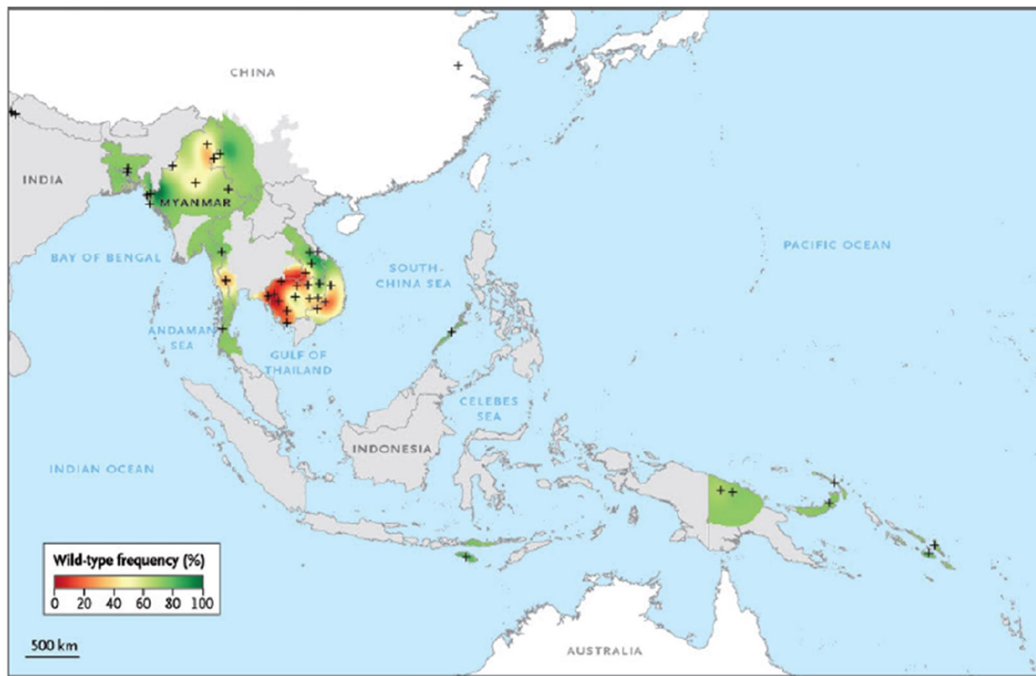

Figure 2.11. Distribution of the Wild-Type K13 Allele in Asia Areas in which malaria is endemic are shaded in gray, and areas that are considered to be malaria-free are shown in white. The mean frequency of the wild-type allele is indicated by the color code (Menard *et al.* 2016).

2.4.4.3 Scenario in the areas of India and Myanmar bordering Bangladesh

Tun *et al.* (2015) conducted a cross-sectional molecular survey in Myanmar including regions bordering with Thailand and Bangladesh, between January, 2013, and September, 2014, aimed to assess the spread of artemisinin-resistant *P falciparum* in Myanmar by determining the relative prevalence of *P falciparum* parasites carrying K13-propeller mutations. Three seventy one (39%) of their 940 samples carried a K13-propeller mutation. They recorded 26 different mutations, including nine mutations not described previously in south-east Asia. In seven (70%) of the ten administrative regions of Myanmar, the combined K13-mutation prevalence was more than 20%. Geospatial mapping showed that the overall prevalence of K13 mutations exceeded 10% in much of the east and north of the country. In Homalin, Sagaing Region, 25 km from the Indian border, 21 (47%) of 45 parasite samples carried K13-propeller mutations.

Sequence analysis of *pfk13* from codon 427 to 727 of 384 samples from northeastern Indian states of Arunachal, Tripura, Mizoram, Gujarat, Maharashtra, Madhya Pradesh and West Bengal detected 4 non-synonymous (G533A, S549Y, R561H, A578S) and 2 synonymous mutations and 1 deletion. However, presence of these mutations did not correlate with ACT treatment failures (Mishra *et al.* 2015).

Zaw et al. (2018) reviewed literatures on k13mutant alleles for artemisinin resistance in P. falciparum and relevant literatures published between April, 2015 and December, 2016. They found in four study areas of China-Myanmar border Hainan, Tengchong, Xishuangbanna and CM border, more than 50% of the isolates in CM border carried the k13 mutations whereas 12% and 4.9% of the isolates in Hainan and Xishuangbanna respectively had these mutations. F446I NS mutation was identified in the India-Myanmar border region. This was the first observation of F446I in northeast India. The mutant has been observed to be associated with delayed parasite clearance. This mutant was absent across the India–Bangladesh border, an information consistent with the absence of k13 mutant parasites in Bangladesh. A578S mutant has been reported from Mizoram adjacent to Bangladesh. However, this mutation has been no correlation with treatment outcome in the previous studies. A578S mutant has been reported with no available data on clinical outcome in Bangladesh. A578S mutation is not associated with ART-R whereas it was commonly reported in African countries in a recent study. To date, 13 non-synonymous mutations of k13 gene have been observed to have slow parasite clearance. Worldwide mapping of k13 mutant alleles have shown mutants associated with artemisinin resistance were confined to southeast Asia and China and did not invade to African countries.

In Eastern India, Das *et al.* (2018) followed up 226 falciparum malaria patients for 42 days treated with artesunate-sulfadoxine-pyrimethamine (ASSP) therapy in 2016. They assessed the ASSP treatment efficacy by evaluating parasite clearance half-life, mutation in *pfkelch13* gene and survival of parasites as detected by an ex vivo ring-stage survival assay (RSA). Slow-clearing infections with longer parasite clearance half-lives (>5 hours) were observed in 12% isolates. ASSP failure was recorded in

15.9% isolates. In 10.6% patients, parasite clearance half-life was greater than 5 hours with *pfkelch13* polymorphism after 441 codon; in 15 of those patients (6.6%), parasites had not cleared by 72 hours after initiation of therapy. Median ex vivo ring-stage survival rate of these isolates was much higher (12.2%) than that of cured patients (0.9%). Of these 15 patients, 13 patients had *pfkelch13* G625R polymorphism, whereas 2 patients contained R539T polymorphism. As per the World Health Organization guideline, these 15 isolates were true artemisinin-resistant isolates.

2.4.4.4 Bangladesh scenario

Ministry of Health of Bangladesh, changed the national treatment guidelines to artemisinin-based combination therapies in 2004. Haque *et al. (*2007) conducted a study to determine the baseline therapeutic efficacy of artemether lumefantrine used as a six-dose regimen for the treatment of uncomplicated *P. falciparum* malaria. Sixty-seven patients were enrolled in the study; the cure rate in a 42-day follow-up after an adjustment by polymerase chain reaction was 94.3%. These data suggest that artemether-lumefantrine is a highly efficacious and well-tolerated treatment for uncomplicated *P. falciparum* malaria in Bangladesh.

Samad *et al.* (2013) conducted an open randomized controlled trial to compare the efficacy of two fixed dose combinations of artemesinin based combinations for uncomplicated falciparum malaria in Bangladesh. A total of 252 cases were randomized to receive Artesunate + Amodiaquine (AA group, 147 cases) and Artemether + Lumefantrene (AL group, 106 cases). The distribution of the cases was comparable by age, sex and study sites. Treatment success' response was observed 100% in the AL group and AA group had 99%, two failures with AA were late treatment failures and the difference was not statistically significant ($p > .1$). The parasitological sensitive (S) response was observed in 97% of cases in AL group and 95% in the AA group, and was not a statistically significant difference. There was no significant difference in parasite clearance time between two groups of cases. The two

ACT regimen, AA and AL had no significant difference in efficacy and safety for treatment of Uncomplicated Malaria in Bangladesh.

Mohon *et al.* (2014) sequenced K13 propeller gene from 253 microscopically positive *P. falciparum* samples originated from endemic districts of Bangladesh and detected a non-synonymous mutation (A578S) among two samples from Matiranga area of Khagrachari district. This is the only report of any K13 mutation detected so far in samples from Bangladesh.

A molecular analysis of 130 *P. falciparum* isolates collected between August 2014 and January 2015 from Bandarban, an endemic district of Chittagong Hill Tracts, Bangladesh revealed no single nucleotide polymorphisms in the *k13* propeller region. (Alam *et al.* 2017).

2.4.4.5 Scenario in Africa and other regions

In a study conducted by Ashley *et al.* (2014), enrolled 1241 adults and children between May 2011 and April 2013, with acute, uncomplicated falciparum malaria in an open-label trial at 15 sites in 10 countries (7 in Asia and 3 in Africa). The researchers found the median parasite clearance half-lives ranged from 1.9 hours in the Democratic Republic of Congo to 7.0 hours at the Thailand–Cambodia border. Slowly clearing infections (parasite clearance half-life >5 hours), strongly associated with single point mutations in the “propeller” region of the *P. falciparum* kelch protein gene on chromosome 13 (*kelch13*), were detected throughout mainland Southeast Asia from southern Vietnam to central Myanmar. The incidence of pretreatment and post-treatment gametocytemia was higher among patients with slow parasite clearance, suggesting greater potential for transmission. In western Cambodia, where artemisinin-based combination therapies are failing, the 6-day course of antimalarial therapy was associated with a cure rate of 97.7% (95% confidence interval, 90.9 to 99.4) at 42 days.

Madamet *et al.* (2015) investigated *P. falciparum* isolates collected from 29 malaria patients treated with artemether-lumefantrine between 2013 and 2014 in Mayotte, a

French island located in the Indian Ocean in the Comoros archipelago. Seventeen percent of the isolates presented mutations in one of the six K13-propeller blades (N490H, F495L, N554H/K, and E596G). A total of 23.8% of the isolates from the Union of Comoros showed K13-propeller polymorphisms. Three of the 18 isolates (16.7%) from Grande Comore showed polymorphisms (N490H, N554K, and E596G).

As per the worldwide map of *P. falciparum* K13-propeller polymorphisms, constructed by Menard *et al.* (2016) in Africa, no Asian artemisinin-resistance allele was observed among 9542 sequences, but 150 distinct alleles were identified, 92% of which were found in only one or two samples. Apart from A578S, V589I, S522C, V534A, F583L, and G665C, most alleles were Africa-specific and localized. A578S, which ranked fourth in abundance among mutant isolates, was observed in 1 sample from Thailand and 41 samples from Africa (Figure 2.12).

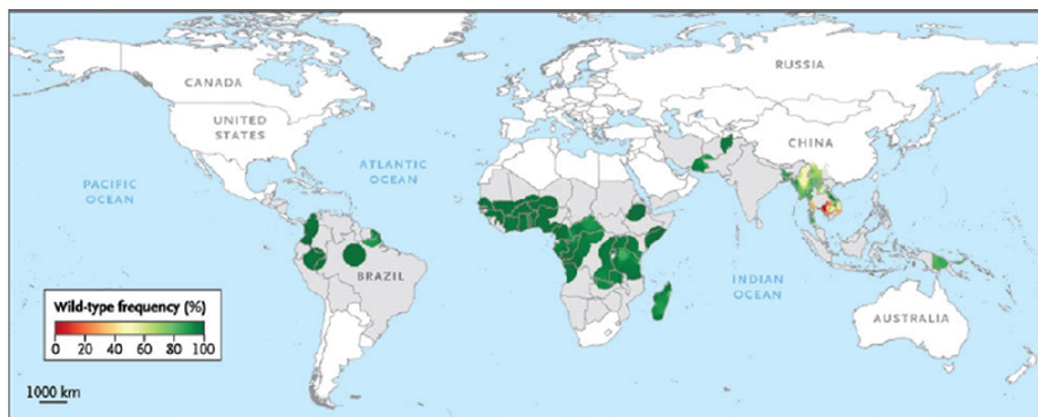

Figure 2.12. Global distribution of the Wild-Type K13 Allele. (Menard *et al.* 2016)

To clarify whether *Pfkelch13* mutations have been selected by artemisinin usage or merely reflect natural polymorphism independent of selection, Mita *et al.* (2016) sequenced the *Pfkelch13* propeller domain in 581 isolates collected before (420 isolates) and after (161 isolates) the implementation of artemisinin combination therapies (ACTs), from various regions of endemicity worldwide. Nonsynonymous mutations were observed in 1% of parasites isolated prior to the introduction of ACTs. Frequencies of mutant isolates, nucleotide diversity, and haplotype diversity were

significantly higher in the parasites isolated from populations exposed to artemisinin than in those from populations that had not been exposed to the drug. In the artemisinin-exposed population, a significant excess of dN compared to dS was observed, suggesting the presence of positive selection. In contrast, pairwise comparison of dN and dS and the McDonald and Kreitman test indicate that purifying selection acts on the *Pfkelch13* propeller domain in populations not exposed to ACTs.

In Africa, non-synonymous mutations are still rare and highly diverse. Non-synonymous K13 mutations have been reported in Angola, Benin, Burkina Faso, Cameroon, Central African Republic, Comoros, Congo, Cote d' Ivoire, Democratic Republic of the Congo, Equatorial Guinea, Eritrea, Ethiopia, Gabon, Gambia, Kenya, Liberia, Madagascar, Malawi, Mali, Mozambique, Niger, Nigeria, Rwanda, Senegal, Somalia, Sudan, Sierra Leone, Tanzania, Togo, Uganda, Zimbabwe and Zambia. The most frequent allele observed in Africa has been A578S, although it has not been associated with clinical or *in vitro* resistance to artemisinin. A number of mutations, including some associated with delayed clearance in the GMS (in particular C580Y), have been reported in Africa (WHO 2018[d]).

C580Y haplotypes have also been reported in Equatorial Guinea and Ghana, in Chinese travelers returning to their country. These mutations most probably emerged in Africa. Similarly, C580Y mutations have been found in samples from Papua New Guinea and Guyana what are believed to be local strains. None of these four countries have reported treatment failures linked to C580Y with an ACT (WHO 2018[d]).

In 282 *P. falciparum* cases returned from Africa to Shandong province of China between 2012 and 2015 Xu C *et al.* (2018) identified 1 synonymous and 9 non-synonymous mutations in *Pfkelch13* (4.6%), among which a candidate artemisinin (ART) resistance mutation P553L was observed.

Mvumbi *et al.* (2017) investigated resistance to artemisinin-based combination therapies currently in use in Democratic Republic of Congo by surveying molecular polymorphisms in three genes: *pfcrt, pfmdr1* and *pfk13*. Mutations in *pfcrt, pfmdr1*

and *pfk13* genes were found in 63.9%, 1.07% and 3.4% samples respectively. They identified 9 samples with mutations in the propeller domain of the K13 among which 3 mutations previously described (F495L, S522C and V520A) and 3 new mutations (M476K, N523T and E509D). None of the mutations found in South-East Asia correlated with artemisinine resistance have been found in Democratic Republic of Congo.

In D R Congo Mayengue *et al.* (2018) amplified k13 propeller region of *P. falciparum* DNA by a nested PCR from 145 samples from Brazzaville and sequenced 127 out of them. They found none of the mutations that were associated with ACTs resistance in Southeast Asia. However, one mutation was observed at position 578, where alanine was substituted by serine (A578S) in two isolates.

In a systematic review of related studies published since 2014 Ocan *et al.* (2019[b]) found the aggregate prevalence of SNPs in *pfkelch13* gene to be 27.6%. Sub-group analysis showed that aggregate prevalence of non-synonymous SNPs in *pfk13* gene was higher (45.4%) in Southeast Asia as opposed to 7.6% in the African region. They identified 165 independent *k13* mutations across malaria-affected regions globally, over half were reported as new alleles. A total of 16 non-validated *k13* mutations were associated with increased ART parasite clearance half-life ($t_{1/2} > 5$ h). They also identified 20 non-propeller mutations in the *pfkelch13* gene.

2.5 Therapeutic outcome in malaria treatment

2.5.1 Outcome of common antimalarial drugs

Since the development of widespread resistance to chloroquine around the world, therapeutic efficacy study of different antimalarial drugs and their combinations has been almost a routine affair for decades. Studies in South East Asia and Sub Saharan Africa shows variable range of efficacy of common antimalarial drugs other than artemisinin and its combinations.

A study in Myanmar the next door neighbor of Bangladesh assessed clinical and parasitological responses on day 3 and days 4±14 of antimalarial therapy to see early and late treatment failure (Ejov *et al.* 1999). Mefloquine was five times more likely to be effective than chloroquine and sulfadoxine-pyrimethamine (S-P), whereas chloroquine and S-P treatments had nearly identical failure patterns. The alarming frequency of clinical and parasitological failure (failure rate >50%) following chloroquine treatment was reported in Sagaing and following S-P treatment in Sagaing and eastern Shan province.

In Thailand evidence of declining efficacy of quinine in severe falciparum malaria was observed before introduction of artemisinin (Pukrittayakamee *et al.* 1994). Parasite clearance time was observed to exceed 96 h in 33% (sol2678) of patients compared with 14% (sol15102) previously ($P = 0 \cdot 006$). Quinine remained an effective treatment for severe multi-drug resistant falciparum malaria in that area, but there was evidence of a decline in the immediate therapeutic response.

A combination of quinine and clindamycin was found safe and effective in treating multidrug-resistant falciparum malaria in Thailand (Pukrittayakamee *et al.* 2000). In that study total 204 patients were randomized to receive a 7-day oral treatment regimen of quinine (Q7) either alone (*n* 5 68), in combination with clindamycin (Q7C7; *n* 5 68), or in combination with tetracycline (Q7T7; *n* 5 68). All patients had uncomplicated recoveries with no serious adverse effects. Fever clearance times for both of the two combination regimens were significantly shorter than that for the Q7-

only. Parasite clearance times were not significantly different between the three treatment groups. The cure rates assessed at 28 days of follow-up were 100% for Q7C7 and 98% for Q7T7, whereas the cure rate was 87% for the Q7-only regimen.

Achan *et al.* (2009) studied ffectiveness of quinine versus artemether-lumefantrine for treating uncomplicated falciparum malaria in 175 Ugandan children in a randomized, open label effectiveness study between 2007 and 2008. Using survival analysis the cure rate unadjusted by genotyping was 96% for the artemether-lumefantrine group compared with 64% for the quinine group (P=0.001). In the quinine group 69% (18/26) of parasitological failures were due to recrudescence compared with none in the artemether-lumefantrine group. The mean adherence to artemether-lumefantrine was 94.5% compared with 85.4% to quinine (P=0.0008).

Yeka *et al.* (2013) also studied efficacy of quinine, artemether-lumefantrine and dihydroartemisinin-piperaquine as rescue treatment for uncomplicated malaria among 220 Ugandan children between 2007 and 2009. The risk of recurrent infection was significantly higher in patients treated with quinine (70 %) and AL (60%) compared to DHAPQ (25%). Recrudescence tended to be lower in the DHAPQ (1%) than in the quinine (7%) or AL (6 %) group.

However, high efficacy of artemether lumifrantrine combination in treating uncomplicated *P. falciparum* malaria is observed in majority of the endemic areas. In Ethiopia Nega *et al.* (2016) followed 91 *P. falciparum* patients for 28 days between October 2014 and January 2015 who received standard six-dose regimen of AL was over three days. Of the 91 patients enrolled, the day-28 analysis showed 83 adequate clinical and parasitological responses (ACPRs). Per protocol analysis, PCR-uncorrected and corrected cure rates of Coartem1among the study participants were 97.6% (95%CI: 93.6–99.5) and 98.8% (CI 93.5–100%), respectively. No parasite was detected on day 3 and onwards. Fever clearance was above 91% on day-3. Mean hemoglobin was significantly increased (P<0.000) from 12.39 g/dl at day 0 to 13.45 g/dl on day 28. No serious adverse drug reactions were observed among the study participant.

No antimalarial prophylactic regimen gives complete protection, but good chemoprophylaxis (adherence to the recommended drug regimen) significantly reduces the risk of fatal disease (WHO 2017).

Ebenebe *et al.* (2018) studied the efficacies of 3-day regimens of artemether-lumefantrine (AL), artesunate-amodiaquine (AA), and dihydroartemisinin-piperaquine (DHP) in 910 children < 5 years old with uncomplicated malaria from six geographical areas of Nigeria. Parasite positivity 1 day and Kaplan-Meier estimated risk of persistent parasitemia 3 days after therapy initiation were both significantly higher, and geometric mean parasite reduction ratio 1 day after treatment initiation (PRRD1) was significantly lower in AL-treated children than in AA- and DHP-treated children. No history of fever, temperature > 38°C, enrollment parasitemia > 75,000 μL^{-1}, and PRRD1 < 5,000 independently predicted persistent parasitemia 1 day after treatment initiation. Parasite clearance was significantly faster and risk of reappearance of asexual parasitemia after initial clearance was significantly lower in DHP-treated children. Overall, day 42 PCR-corrected efficacy was 98.3% and was similar for all treatments. In a non-compartment model, declines of parasitemias were monoexponential with mean terminal elimination half-life of 1.3 hours and unimodal frequency distribution of half-lives. All treatments were well tolerated. In summary, all three treatments evaluated remain efficacious treatments of uncomplicated malaria in young Nigerian children, but DHP appears more efficacious than AL or AA.

2.5.2 Outcome of chemoprophylaxis with mefloquine

Mefloquine is a highly efficacious drug (>90%) against chloroquine-resistant *P. falciparum* malaria, and because of growing chloroquine resistance and mefloquine's widespread market availability since the 1980s, this agent commonly has been used as an alternative to chloroquine in prophylaxis against malaria in most parts of the world. Concerns about potentially severe side effects have been voiced in recent years, even though controlled trials have found the drug to be generally well tolerated, with only mild side effects (Jong and Nothdurft, 2001).

Croft *et al.* (2000) reviewed randomized trials to assess the effects of mefloquine in adult travellers compared to other regimens in relation to episodes of malaria, withdrawal from prophylaxis, and adverse events. They included 10 trials involving 2750 non-immune adult participants. Five of these were field trials, and of these all were in mainly male soldiers. One trial comparing mefloquine with placebo showed mefloquine prevented malaria episodes in an area of drug resistance (Peto odds ratio 0.04, 95% confidence interval 0.02 to 0.08). Withdrawals in the mefloquine group were consistently higher in four placebo controlled trials (odds ratio 3.56, 95% confidence interval 1.67 to 7.60). In five trials comparing mefloquine with other chemoprophylaxis, no difference in tolerability was detected.

Gonzalez *et al.* (2018) reviewed randomized and quasi-randomized controlled trials comparing mefloquine IPT or mefloquine prophylaxis against placebo, no treatment, or an alternative drug regimen. They included six trials conducted between 1987 and 2013 from Thailand, Benin, Gabon, Tanzania, Mozambique, and Kenya that included 8192 pregnant women.When compared with sulfadoxine-pyrimethamine, IPTp-mefloquine resulted in a 35% reduction in maternal peripheral parasitaemia at delivery, but may have little or no effect on placental malaria infections. Mefloquine resulted in little or no difference in the incidence of clinical malaria episodes during pregnancy.

Mefloquine has been associated with rare but serious adverse reactions (such as psychoses or seizures) at prophylactic doses; these reactions are more frequent with the higher doses used for treatment. Other side effects that have occurred in chemoprophylaxis studies include gastrointestinal disturbance, headache, insomnia, abnormal dreams, visual disturbances, depression, anxiety disorder, and dizziness. Other more severe neuropsychiatric disorders occasionally reported during postmarketing surveillance include sensory and motor neuropathies (including paresthesia, tremor, and ataxia), agitation or restlessness, mood changes, panic attacks, forgetfulness, confusion, hallucinations, aggression, paranoia, and encephalopathy (Arguin and Tan, 2018).

2.6 Knowledge attitude and practice (KAP) on malaria

Adequate knowledge on malaria transmission and preventive practice by the people at risk is an important tool in control and elimination of malaria in endemic countries. Studies on KAP on malaria are quite common in both Asia and Africa.

Elsie and Funglada (1994) suggested that, risk factors associated with malaria in Southeast Asian countries can be broadly divided into three groups: (1) social and behavioral risk factors favoring increased occurrence and transmission, *i.e.* poor housing conditions, population movements, irregular or non-use of mosquito nets, partial or non-conformance with residual DDT spraying, etc.; (2) behavioral risk factors predisposing to severe and complicated malaria (not clearly known, probably delayed treatment); and (3) behavioral risk factors related to occurrence of drug resistance, i.e. treatment-seeking patterns, practices of drug utilization and population movements.

Deressa *et al.* (2004) surveyed Knowledge, Attitude and Practice about malaria, vectors and antimalarial drugs in a rural community of Ethiopia. They reported that fever, headaches, chills and shivering were the most frequently mentioned symptoms of malaria reported by 89.7%, 87.5% and 81.3% of the study subjects, respectively. About 66% of the study community related the mode of transmission to the bite of infective mosquitoes and 43.7% of them believed that malaria could be transmitted from person to person through the bite of mosquitoes. It was observed that peoples" knowledge was quite high. For instance, mosquitoes are mainly believed to bite humans at night (73.2%), breed in stagnant water (71%) and rest in dark places inside houses during daytime (44.3%) answered by a considerable number of respondents. Malaria was thought to be preventable by 85.7% of the respondents.

In northern Ghana, Adongo *et al.* (2005) conducted a survey to evaluate the effect of local community knowledge about malaria affects insecticide treated net use. They observed that people recognized the term 'malaria' but had limited biomedical knowledge on the disease, including the etiology, the role of the vector, and host

response. That study also reported the people knowledge on the role for ITNs in nuisance reduction, but not for malaria prevention.

A KAP study to assess the respondents' probable first response after attacked by malaria was conducted by Erhun *et al.* (2006) in Nigeria. Data showed that 35.5%, 0.9% and 13.4% of respondents will use synthetic antimalarials, consult a herbalist and use local herb, respectively, while 27.3%, 1.7% and 31 18.2% will go to the hospital, take spiritual/ritual waters for cure and just pray, respectively, with 3.0% of the respondents indicating that they will ignore the signs. Factors influencing respondents" choice of malaria treatment and preventive methods included cost, religious beliefs, perceived safety, convenience and respondents" state of health for 22.7%, 5.4%, 20.8%, 26.5% and 24.6% of the respondents, respectively. The use of insecticide impregnated net are uncommon amongst the respondents. Treatment seeking practice in malaria was related to level of education and religion. They found that convenience and the severity of the disease affected respondents" choice of treatment in more than 50% of the cases.

A KAP program conducted by Soan and Chand (2006) in tribal population in India demonstrated that only 37.6% of respondents knew that malaria was transmitted by mosquito. Majority of the population (94.2%) were not aware that it was a fatal disease and responded that any person can have malaria. Only 27.2% of respondents suspect malaria on the occurrence of fever. Knowledge about the malarial drug was poor and only 14.6% knew about the drug chloroquine. Smoke was the major preventive measure taken up by the respondents to avoid mosquito bite. Only 5% of the respondents were using mosquito net. Most of the respondent knew about the importance of the insecticide spray and prefer to get only cattle sheds sprayed.

Enato *et al.* (2007) conducted KAP program on malaria management in Nigeria. The study revealed that 87% of the respondents had experienced at least one episode of malaria during their current pregnancy. Most respondents (89%) recognized that they had attributed malaria due to infected mosquito bite, while 75% consider malaria an important health risk during pregnancy. However, knowledge of the consequences of

malaria during pregnancy was poor, especially the risk posed to the fetus. Overall, the mean knowledge score on a scale of „0-7" was 3.5 (median 4.0). Respondents had poor belief in the effectiveness and use of insecticide treated bed nets and intermittent preventive therapy, in preventing malaria during pregnancy.

A two-stage cluster survey on KAP on malaria conducted in Haiti by Keating *et al.* (2008) concluded that respondents in households with more assets were significantly possessed more malaria knowledge than those in households with fewer assets. Their results suggest that malaria control in Haiti should focus on enhanced surveillance and case management, with expanded information campaigns about malaria prevention and treatment options.

In Bangladesh Ahmed *et al.* (2009) conducted a two-stage cluster sampling technique to perceive Knowledge on the transmission, prevention and treatment of malaria among two endemic populations and their health-seeking behavior. Poverty and level of schooling were reported as important determinants of malaria knowledge and practices. Allopathic treatment was uniformly advocated. Of those who sought treatment, the majority went to the village doctors and drugstore salespeople (around 40%). In addition, there was a delay beyond twenty-four hours in beginning treatment of malaria-like fever in more than half of the instances. In the survey, gender divide in knowledge and health-seeking behavior was observed disfavoring women. The respondents in this study were lacking comprehensive knowledge on different aspects of malaria, which was influenced by level of poverty and education. A gender and geographical divide in knowledge was observed disfavoring women and south-eastern area respectively. The participnats preferred allopathic treatment for malaria, although a substantial proportion did not seek any treatment or sought self-treatment for malaria-like fever. This study revealed the care seeking due to malaria was delayed in many occasions.

Adedotun *et al.* (2010) evaluated Knowledge, attitudes and practices about malaria in an urban community in south-western Nigeria. The key findings of their research was about 93.2% (n=192) of respondents recognized mosquito bites as the cause of

malaria. A small proportion of children (13.7%) and adults (5.3%) received prompt treatment; however, more adults (65.8%) got correct dosage of antimalarials than children (38.7%). About 90% of suspected malaria cases in children and adults were first treated at home with local herbs or drugs, purchased from medicine stores. The use of insecticide-treated nets (ITNs) was scarce as only 16.7% of households used them for their children. Other reported malaria prevention methods include the use of insecticides (79.7%) and herbs (44.3%). In all, 17 (8.9%) of households did not have screening nets on their windows and 6.3% of 175 households with screening nets on their windows had rusty and torn nets. The level of education of heads of households was a strong predictor of positive malaria-related KAP. Window types and environmental hygiene were associated with prevalence of malaria in households.

In Bangladesh Haque *et al.* (2010) observed that the most common treatment facility for malaria-associated fever was malaria control programme led by BRAC and Government of Bangladesh (66.6%) followed by the drug vendors (48.8%). Closeness to health facilities runs by the malaria control programme and drug vendors were significantly associated with the choice of treatment. A high proportion of people preferred drug vendors without having a proper diagnosis. They concluded that drug vendors are highly patronized and thus there was a need to improve their services for public health good.

Hossain *et al.* (2010) conducted a study in the Dighinala, Khagrachari of Bangladesh to explore the explanatory model of malarial illness in different communities. This study reported that the knowledge of malaria varies according to ethnicity. The *Chakma* community knows the symptoms of malaria better than the Bangali and the Tripura communities. Most of them knew that mosquito bite was the main cause of the disease and that it was a seasonal disease. Very few possessed in-depth knowledge about the mode of transmission. The perception of malaria including its treatment and prevention has changed over time and this was quite plausible. The community people unanimously perceived allopathic treatment as the „treatment of choice" for curing malaria which makes it much easier for modern malaria prevention and control activities to push ahead.

In Bangladesh Bashar *et al.* (2012) interviewed 468 individuals from individual households. Nearly 86% of the respondents did not know the exact cause of malaria or the role of Anopheles mosquitoes in the pathogen's transmission. Knowledge on malaria transmission and symptoms of the respondents of zones 1 and 2 were significantly ($p<0.01$) different. The majority of respondents believed that bed nets were the main protective measure against malaria. People who spent time outside in the evening were more likely to contract malaria than those who did not.

A community-based cross-sectional study was conducted on a sample of 454 household heads or their deputies to assess the knowledge and practice of malaria prevention methods among the residents of Arba Minch area of Southern Ethiopia (Astatkie, 2010). Majority (86.8%) of the respondents mentioned fever as a symptom of malaria, and 98.2% of the respondents reported mosquito bite as the cause of malaria. Three hundred and eighty four (84.6%) of the respondents mentioned mosquito nets as protective measures against mosquito bites. The protective measure mostly used by the respondents or other household members in the last 12 months was mosquito net (73.3%) followed by aerosol insecticide (13%) with the former being used more in rural areas and the latter in urban areas.

In Uganda a cross-sectional survey revealed that larger proportion (64.6%) of a rural community members had low knowledge on malaria prevention methods, with untreated mosquito nets (81.7%), mosquito coils (36.9%) and insecticide treated nets (29.6%) being the most known methods. Knowledge on malaria prevention methods was associated with age, employment status, education, income and having heard a malaria message in the previous 12 months. Households that had at least one mosquito net were 45.5% and net ownership increased with household income. Only 0.5% of the houses had undergone indoor residual spraying in the previous 12 months, while 2.1% had complete mosquito proofing in windows and ventilators to prevent mosquito entry (Musoke *et al.* 2015).

Bhattacharyya (2015) conducted a KAP survey on malaria in urban setting of East Khasi Hills district, Meghalaya, India. He found that in the community, 92.2% people

had heard of malaria, 86.4% had knowledge that mosquito bites causes malaria, and 53% know that it is preventable. Thirteen percent people said that they used mosquito nets, 42% used mosquito coils, and 41.1% commented that they adopted no measures. Of 13% people using mosquito nets, only 2.9% were found to use insecticide-treated bed nets. Regarding spraying of DDT, only 6.3% people said that DDT was sprayed in their area at least once a year.

A systematic literature review was conducted by Regmi *et al.* (2016) searching six databases, between 1990 and 2015, focusing on knowledge, attitudes and beliefs about malaria in South Asia. General knowledge and awareness of the disease, its transmission, and control and preventative measures were generally found to be lacking amongst both the general public and healthcare professionals. In addition, the study showed that poor socio-economic factors including limited access to services due to poor/limited availability and issues of affordability are considered as major risk factors.

A cross-sectional study involving 400 households in central India revealed that only 50% of the study respondents believed that malaria is fatal disease, only 33% had closed water drainage system in their houses and 62.50% practiced of throwing garbage in open or in-front of their house. Seepage of water was present in 42.37% houses. 15% households did not use any type of personal protective measure (Borkar *et al.* 2017).

A household-based cross-sectional survey on KAP regarding malaria in the south western region of Saudi Arabia bordering with Yemen was conducted by Khairya *et al.* (2017). They found that, majority of respondents (98.4%) reported that they had heard about malaria, but only 21.7%reported that they had sufficient information about the disease. Surprisingly, the most popular source of information was the internet and social media (proportion responding positively in parenthesis) (25.5%), followed by family (21.7%), while information from health facilities contributed only 12.4%. A majority of respondents were aware that malaria is a communicable (89.1%) and deadly (70%) disease; however, only 30.2% of the respondents

responded that malaria is a treatable disease. Almost all of the aware respondents (97.5%) were inclined to seek treatment from health facilities, and 63.2% preferred to seek treatment within 24 h of presenting with symptoms. Regarding personal precautions, the most common practice adopted by respondents was indoor residual spraying IRS (47.3%), followed by anti-mosquito spraying (29.8%), mosquito bed nets (13.2%) and combined anti-mosquito sprays and nets on windows(4.7%).

A KAP survey among non-medical university students in Nigeria found that, less than one-tenth of the respondents (5.4%) knew that *P. falciparum* is the most common species that causes malaria. More than half of the respondents (53.5%) had insecticide-treated mosquito net in their rooms, however only 42.3% of them reported that they sleep under the insecticide-treated mosquito net. There was a strong evidence of association between knowledge of malaria and level of academic study (p=0.015) (Anene-okeke *et al.*2018).

Fernando *et al.* (2016) investigated the knowledge and practices regarding malaria chemoprophylaxis among all the Sri Lankan security forces personnel returning from peacekeeping missions in malaria endemic countries between March and September 2015. Interviews were carried out with 559 security forces personnel returning home from foreign deployments in malaria-endemic regions (males: 550, 98.4 %). The majority (553, 98.9 %) was well aware of the need for chemoprophylaxis during the overseas stay and its regular use as prescribed. The overall adherence to chemoprophylaxis was good with 78.7 % (440/559) reporting regular, as prescribed, use. Having better educational qualifications, being female, being prescribed mefloquine, having fever during deployment and belonging to a security force other than the army were significantly associated with poor compliance ($p < 0.05$).

Hong *et al.* (2017) conducted a study to assess Korean soldier' knowledge on malaria and malaria preventive behavior. Data collected from 294 Korean soldiers nearby the demilitarized zone in Gyeonggi Province in 2016 revealed that, level of education, educational experience on malaria and ranks like corporal, and sergeant were

observed to be associated with the level of knowledge of malaria. Knowledge of malaria and malaria education experience were the factors that influenced malaria prevention behavior and practice.

Tizifa *et al.* (2018) reviewed the prevention and control strategies for malaria, to assess their impact towards reducing the disease burden and to highlight the best practices observed. They noted that use of long-lasting insecticide-treated nets and indoor residual spraying had resulted a decline in the incidence and prevalence of malaria in Sub-Saharan Africa. Other strategies such as larval source management had been shown to reduce mosquito density but require further evaluation. New methods under development such as house improvement had demonstrated to minimize disease burden but require further evidence on efficacy.

A cross sectional study was conducted by Khanam *et al.* (2018) to explore the gap between coverage, access, and utilization of long-lasting insecticide-treated nets (LLINs) among the households of malaria endemic districts in Bangladesh. Among 2640 total households, 77.4% possessed at least two LLINs and 56.8% had insufficient access. Members of 77.9% households had used LLINs the previous night and 6.0% did not use LLINs despite having sufficient access. LLIN use was lower in non-hill track areas, in Bengali community, in richer households and households with more than four members. Their qualitative findings revealed that the major reasons behind not using LLINs were insufficient access, sleeping outside the home, migration, perceived low efficacy of LLINs, or fear of physical side effects.

2.7 Aims and objectives of the research work

2.7.1 General aim

To investigate and compare molecular pattern and epidemiological aspects of anti-malarial drug resistance of *P. falciparum* in members of Armed Forces of Bangladesh working in endemic areas at home and Africa.

2.7.2 Specific objectives

a. To investigate the pattern of genetic polymorphisms in the following genes of *P. falciparum* associated with anti-malarial drug resistance, isolated from members of armed forces working in Chittagong Hill Tracts:
   - *P. falciparum* chloroquine resistance transporter gene (*Pfcrt*) for chloroquine resistance.
   - *P. falciparum* multidrug resistance 1 gene (*Pfmdr1*) for mefloquine and lumefantrine resistance
   - *P. falciparum* kelch13 gene (*pfk13*) for artemisinin resistance

b. To investigate the pattern of genetic polymorphisms in the above mentioned genes, isolated from Bangladeshi troops working in African countries.

c. To compare the prevailing molecular pattern of anti-malarial drug resistance of *P. falciparum* in those two groups.

d. To examine epidemiological aspects of members of Bangladesh Armed Forces suffering from *P. falciparum* malaria in Chittagong Hill Tracts.

e. To examine epidemiological aspects of Bangladeshi troops suffering from *P. falciparum* malaria in endemic countries of Africa.

3.0 Materials and Methods

In this study number of established techniques and procedures were employed to investigate and compare molecular pattern and epidemiological aspects of anti-malarial drug resistance of *P. falciparum* in members of Armed Forces of Bangladesh working in endemic areas at home and Africa. Here is a brief description of the materials and methods followed in the study.

3.1 Ethical statement

Ethical clearance was obtained from The Ethical Committee of Armed Forces Medical Services of Bangladesh which is a body of Directorate General of Medical Services (DGMS), Ministry of Defense of Bangladesh (DGMS letter number: 4614/35/T/DGMS/Ethi, dated 01 October 2014). Informed consent was also obtained from all participants.

3.2 Study place and population

Bangladeshi troops working in Chittagong Hill Tracts (CHT), Bangladesh and five Sub-Saharan African countries were recruited in this study basing on some specific selection criteria.

3.2.1 Inclusion criteria:

(a) Willing member of Bangladesh Armed Forces

(b) Uncomplicated *P. falciparum* malaria case

(c) Diagnosis confirmed by either microscopy or RDT

3.2.2 Exclusion criteria:

(a) Mixed malaria case

(b) 1st sign/symptom appeared within 2 weeks of entering endemic area.

3.2.3 Study sites

3.2.3.1 Bangladesh

Malaria endemic Rangamati, Khagrachari and Bandarbans districts of CHT and Ramu sub-district of Cox's Bazar, were selected as study sites in Bangladesh and military troops working there were study subjects. These places has porous borders with endemic parts of India and Myanmar (figure 3.1).

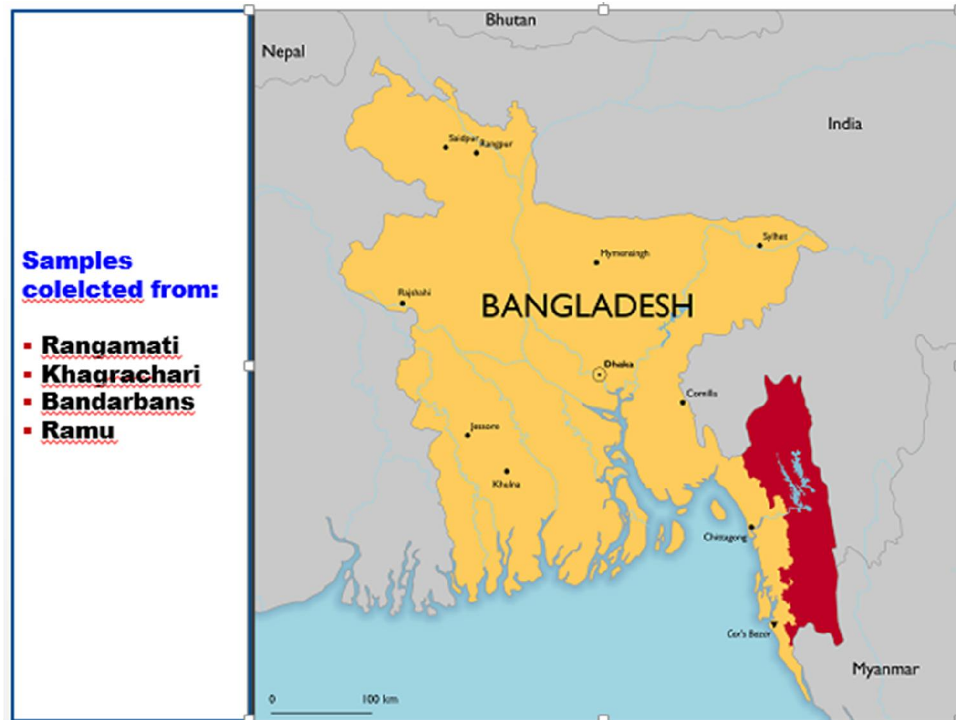

Figure 3.1. Map of Bangladesh showing study sites

3.2.3.2 Africa

Five Sub-Saharan malaria endemic countries namely Mali, Liberia, Ivory Coast, Cenrral African Republic (CAR) and Democratic Republic of Congo (DRC), whre there is deployment of Bangladeshi troops were selected as study sites (figure 3.2). These are included in the African countries where malaria continues to be one of the top killars.

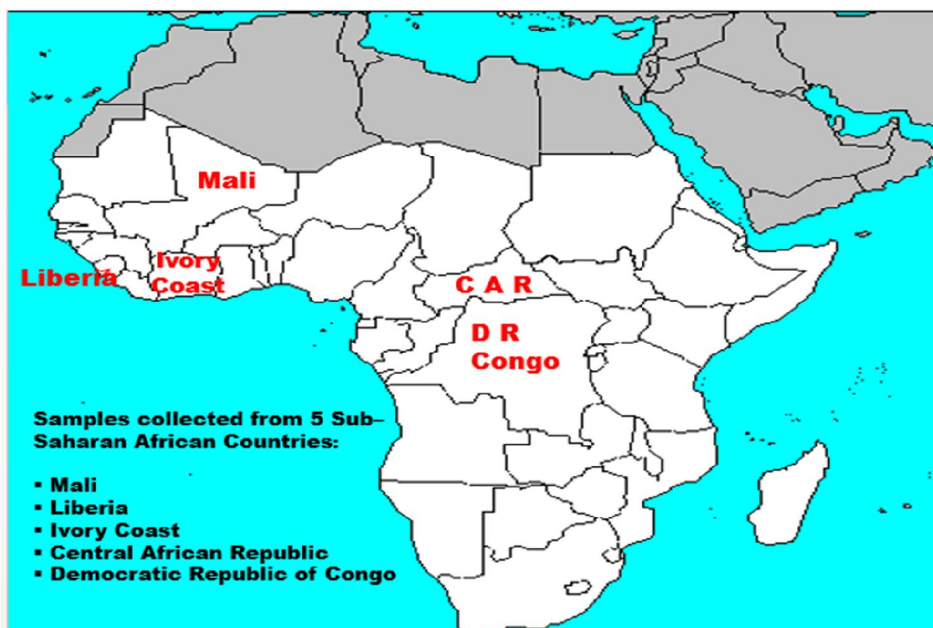

Figure 3.2. Map of Africa showing countries where this study was conducted

3.3 Study period

This study commenced in November 2014 and continued till the end of 2018.

3.4 Training of doctors and paramedics

Doctors and paramedics involved in collection of blood sample and data were trained and provided with necessary filter paper, kit, accessories and copies of data collection instrument before leaving for endemic areas of Bangladesh and Africa.

3.5 Sample collection

After obtaining informed consent, samples were collected from *P. falciparum* malaria confirmed (either by microscopy or RDT) Bangladeshi troops working in CHT, Bangladesh, and five Sub-Saharan African countries. A written instruction describing the procedure of collection of blood samples on filter paper was provided to the sample collectors. The confirmation of *P. Falciparum* by microscopy or RDT in the patients was done by beforehand the treating health facility. We collected blood samples from an individual soldier once he or she was labeled as a case of malaria after either microscopy or RDT done by the treating health facility.

Three spots of finger prick blood were collected on a filter paper (Whatman UK) from every *P. falciparum* malaria patient before starting anti-malarial drug. It was then dried in the air for about half an hour in room temperature and preserved in a zip locked poly-bag with silica pack inside before final transportation to the laboratory of Department of Microbiology, University of Dhaka. In the laboratory these samples were stored in 4^0 c refrigerator.

3.6 Collection of clinical and epidemiological data

A structured questionnaire (appendixI) and a checklist was used to collect relevant clinical, demographic and epidemiological data. Data were collected by face to face interview with the patients and also from their clinical records. The questionnaire and checklist were finalized after being pretested among Bangladeshi troops working in an endemic area of Bangladesh.

Data collection on 'incidence of malaria under mefloquine prophylaxis' was carried out for one year in a Bangladeshi military contingent of 738 men deployed under United Nations peace keeping mission in Liberia.

3.7 Extraction of DNA of *P. falciparum*

P. falciparum DNA extraction was done from all 252 samples at the Microbiology Laboratory, University of Dhaka, using QIAamp® DNA Mini Kit (Qiagen GmbH, Germany 2015), following manufacturer's protocol of DNA Purification from Dried Blood Spots. The procedure is described in brief as follows:

a) Three mm (1/8 inch) diameter punches were cut from a dried blood spot with a single-hole paper puncher. Then 5 punched-out circles were placed into a 1.5 ml micro-centrifuge tube and 180 µl of Buffer ATL was added.
b) The solution was incubated at 85°C for 10 min and briefly centrifuged to remove drops from inside the lid.
c) Twenty µl proteinase K stock solution was added, mixed by vortexing, incubated at 56°C for 1 h and then centrifuged briefly to remove drops from inside the lid.

d) Two hundred µl Buffer AL was added to the sample, mixed thoroughly by vortexing, incubated at 70°C for 10 min and centrifuged briefly to remove drops from inside the lid.

e) Two hundred µl ethanol (96–100%) was added to the sample, mixed thoroughly by vortexing and centrifuged briefly to remove drops from inside the lid.

f) The mixture from step 5 was carefully applied to the QIAamp Mini spin column (in a 2ml collection tube) without wetting the rim. Closing the cap, the mixture was centrifuged at 8000 rpm (6000 X g) for 1 min. Then the tube containing filtrate was discarded leaving the QIAamp mini spin column in a clean 2 ml collection tube.

g) By carefully opening the QIAamp mini spin column, 500 µl Wash Buffer (AW1) was added without wetting the rim. Closing the cap, the column was centrifuged at 8000 rpm (6000 X g) for 1 min. Then the tube containing filtrate was discarded leaving the QIAamp mini spin column in a clean 2ml collection tube.

h) By carefully opening the QIAamp mini spin column, 500 µl Wash Buffer (AW2) was added without wetting the rim. Closing the cap, the column was centrifuged at 14000 rpm (20000 X g) for 1 min. Then the tube containing filtrate was discarded leaving the QIAamp mini spin column in a clean, autoclaved 1.5ml micro-centrifuge tube.

i) By carefully opening the QIAamp mini spin column, 200 µl Elution Buffer (AE) was added without wetting the rim. The column left for incubation at room temperature for about 15 minutes. Then the column was centrifuged at full speed (8000 rpm, 6000 X g) for 1 min.

j) Then the column was discarded leaving the filtrate containing the DNA in the micro-centrifuge tube. The DNA was stored at -20ºC until its use.

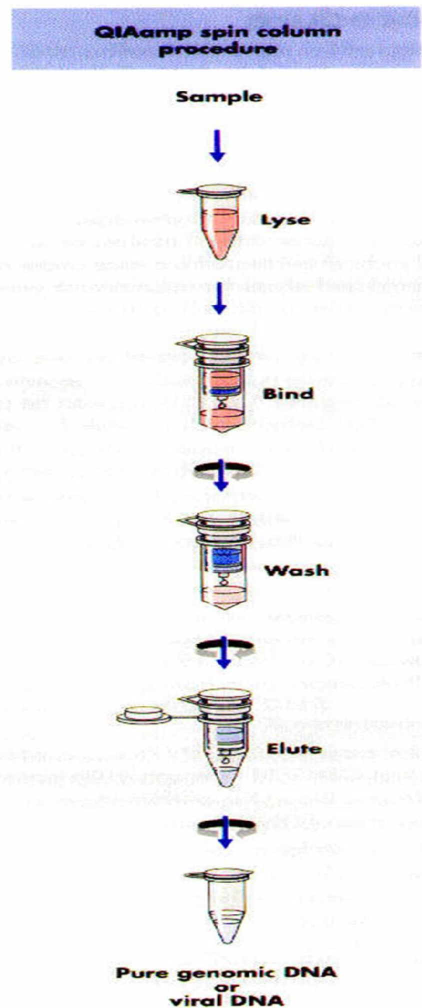

Figure 3.3 DNA extraction flow chart (Qiagen, 2015)): Purified DNA, thus extracted from all 252 dried blood samples was then stored under minus 20° C.

3.8 Important equipment and materials used in all PCR, gel-electrophoresis, RFLP and related procedure

- Applied Bio-system 2720 Thermal Cycler, Thermo Fisher Scientific, USA for all PCR procedures
- Gel-electrophoresis Unit, Sigma, USA
- UV Transilluminator, Vilber, Lourmat, France
- ABI 3500 Genetic Analyzer, Life Technologies for sequencing

3.9 Species identification of *P. falciparum*

P. falciparum species was confirmed by a nested PCR following a standard protocol (Snounou *et al.*, 1993) with minor modifications. This nested based PCR targeted the 18 small subunit ribosomal RNA (ssr-RNA) gene amplifying a 1.2kb fragment of *pf* DNA. Two genus-specific primers rPLU5 and rPLU6, were used for the first cycle of amplification. An aliquot of the product thus obtained, was used as template for a second amplification cycle, in which parasite species was confirmed using species specific primers (rFAL1 and rFAL2). The expected final band size was 205 bp.

3.9.1 Primers for species identifications

a) Two genus specific primers were used to amplify 18ssrRNA gene in step-1:

rPLU5 CCTGTTGTTGCCTTAAACTTC

rPLU6 TTAAAATTGTTGCAGTTAAAACG

b) In step-2, species specific primers were used to amplify size specific portions of the ssrRNA sequence of step 1 that corresponds to *P. falciparum* :

rFAL1 TTAAACTGGTTTGGGAAAACCAAATATATT

rFAL2 ACACAATGAACTCAATCATGACTACCCGTC

Primers were constituted at Bioneer Corporation, Republic of Korea.

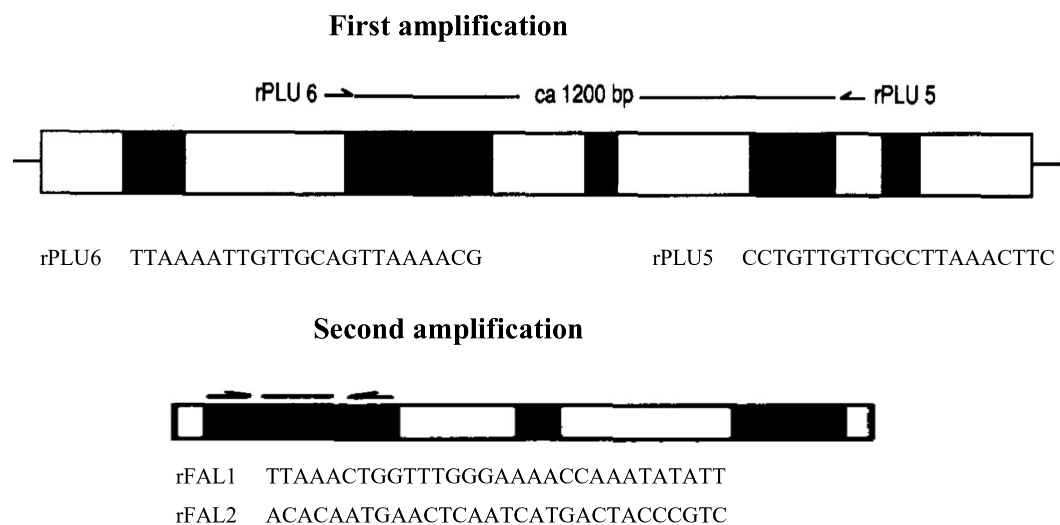

Figure 3.4 Schematic representation of Plasmodium ssrRNA genes and the nested PCR protocol used (Snounou *et al.*, 1993)

3.9.2 PCR mixture and thermal cycling

Step-1 PCR for species confirmation

In step-1, PCR reactions were carried out in a volume of 20 µL. In all cases amplification was performed in in a mixture of reagent, primars (rPLU5 and rPLU6), template DNA and Taq DNA Polymerase (Invitrogen, Thermo Fisher Scientific, USA) as listed in Table 3.1. A seven-step thermal cycling as narrated in table 3.1 was performed in 'Applied Bio-system 2720 Thermal Cycler' of Thermo Fisher Scientific, USA. A '*pf* DNA positive' sample (sample code A63 and B7 confirmed to have *pf* DNA at Parasitology Laboratories, ICDDR,B, Dhaka) were used as positive control. A negative control (without template DNA) was also used in each batch of PCR essay.

Table 3.1. PCR mixture and thermal cycling in step-1

Name of the reagents	Vol. x 1 sample (µL)	Final concentration	Thermal cycling	
10x Standard Taq Buffer	2.0	1 x	step 01	95 °C for 5min
2.5 mM dNTPs	1.0	125 nmol	step 02	94 °C for 1 min
50 mM MgCl2	0.4	2.5 µM	step 03	58 °C for 2 min
10 µM rPLU 5	0.5	250 nmol	step 04	72 °C for 2 min
10 µM rPLU 6	0.5	250 nmol	step 05	go to step 02 (34 cycles)
Taq DNA polymerase	0.1		Step 06	58 °C for 2 min
PCR grade H2O	13.5		Step 07	72 °C for 7 min
Template DNA	2		Final hold	4 °C
Total	20.0			

Step-2 PCR (nested) for species confirmation

The volume of PCR reaction in step-2 was also 20 µL. Constituents of PCR mixture and steps of thermal cycling are enumerated in Table 3.2. A 2 µL aliquot from the product of the first PCR reaction was used as a template and a pair of species-specific primers were employed in step-2. Expected product size was 205 bp denoting *P. falciparum* species.

Table 3.2. PCR mixture and thermal cycling in step-2 (nested)

Name of the reagents	Vol. x 1 sample (µL)	Final concentration	Thermal cycling	
10x Standard Taq Buffer	2.0	1 x	step 01	95 °C for 5min
2.5 mM dNTPs	1.0	125 nmol	step 02	94 °C for 1 min
50 mM MgCl2	0.4	2.5 µM	step 03	58 °C for 2 min
10 µM rFAL1	0.5	250 nmol	step 04	72 °C for 2 min
10 µM rFAL2	0.5	250 nmol	step 05	go to step 02 (39 cycles)
Taq DNA polymerase	0.1		Step 06	58 °C for 2 min
PCR grade H2O	13.5		Step 07	72 °C for 7 min
Template DNA	2		Final hold	4 °C
Total	20.0			

3.9.3 Gel electrophoresis

The products of step-2 PCR were analyzed by electrophoresis on a 2% agarose gel stained with ethidium bromide (Et-Br) and visualized under UV trans-illumination. Size of PCR products were estimated using 100 base pair (bp) DNA ladder marker.

Procedure

A 2% agarose gel was prepared by dissolving agarose (Sigma, USA), in 1X Tris-borate EDTA (TBE) buffer. Then, it was heated for 2-3 minute, cooled to 50°C, stained with 2 µl Et-Br and poured into gel electrophoresis unit (Sigma, USA) with spacers and comb. After solidification of the gel, the comb was removed and the gel was submerged in 1X TBE buffer in the gel dock. Five µl of each PCR product, mixed with 1 µl gel loading dye was poured into each well. Marker DNA of known size (100bp ladder) (Bioneer, Republic of Korea) was loaded in one well. Electrophoresis was carried out at 95 volts for approximately 45 minutes. The DNA bands intercalated with Et-Br were observed on a UV transilluminator (Vilber Lourmat, France). Photographs were taken using the gel documentation system of the unit.

3.10 Genotyping of *pfcrt* and *pfmdr1* genes of *P. falciparum* for drug resistance status

Antimalarial drug resistance has been associated with the presence of single nucleotide polymorphisms (SNPs) in particular gene of *P. falciparum.* (Veiga et. al. 2006) These include:

a) The *pfcrt* (*P. falciparum* chloroquine resistance transportes) K76T a.a. change is correlated to chloroquine and amodiaquine resistance.

b) *pfmdr1* (*P. falciparum* multidrug resistance 1) N86Y alternation is associated with resistance to mefloquine and lumefantrine.

The presence of the *pfcrt* K76T and *pfmdr1* N86Y mutations were assessed in the isolates using Multiplex PCR–RFLP method, following the protocol of Veiga *et al.* with minor modifications.

3.10.1 Multiplex nested PCR

A set of first and internal (nest) primers were used to amplify DNA fragments containing *pfmdr1* N86Y and *pfcrt* K76T. Primers were synthesized at Bioneer Corporation, Republic of Korea. After 1st round amplification amplicon from the 1st step was used as template for 2nd round amplification. The expected final band size of *pfmdr1* and *pfcrt* were 418 bp and 145 bp respectively (Table 3.3).

Table 3.3. Sequence of the primers used for amplification of *pfcrt* and *pfmdr1* genes by multiplex nested PCR

Steps	Alleles	Primers	Sequence (5' - 3')	Amplicon size
Step-1	*pfmdr1*	M1 FW	AAGAGGTTGAAAAAGAGTTGAAC	447 bp
		M1 REV	CCGTTAATAATAAATACACGCAG	
	Pfcrt	C1 FW	ATTTCGTACCAATTCCTGAACT	538 bp
		C1 REV	CGGATGTTACAAAACTATAGTTACC	
Step-2 (nested)	*Pfmdr1*	M2 FW	AGAGTACCGCTGAATTATTTAG	418 bp
		M2 REV	CCTGAACTCACTTGTTCTAAAT	
	Pfcrt	C2 FW	TGTGCTCATGTCTTTAAACTT	145 bp
		C2 REV	CAAAACTATAGTTACCAATTTTG	

Step-1 PCR for Genotyping of *pfcrt* and *pfmdr1* genes

PCR reactions were carried out in a volume of 25 µL. Purified DNA was used as template. Ingredients of the reaction mixture are listed in Table 3.4. Taq DNA Polymerase, used in these reactions was 'Maximo Taq DNA Polymerase', GeneON GmbH, Germany. For amplification of the target gene, a seven-step thermal cycling was performed as narrated in Table 3.4.

Table 3.4. Reaction mixture and thermal cycling in step- 01

Name of the reagents	Vol. x 1 sample (µL)	Final concentration	Thermal cycling	
10x Standard Taq Buffer	2.5	1 x	step 01	94 °C for 3min
10 mm dNTPs	1.0	0.4 mM	step 02	94 °C for 30 sec
50 mM MgCl2	1.5		step 03	56 °C for 30 sec
M1 Fw	2.0	0.8 µM	step 04	68 °C for 30 sec
M1 Rev	2.0	0.8 µM	step 05	go to step 02 (45 cycles)
C1 Fw	3.0	1.2 µM	step 06	68 °C for 3 min
C1 Rev	3.0	1.2 µM	Final hold	12 °C
Taq DNA polymerase	0.3			
PCR grade H2O	4.7			
Template DNA	5.0			
Total	25.0			

Step-2 PCR (nested) for Genotyping of *pfcrt* and *pfmdr1* genes

In step-2, PCR reaction volume was also 25 µL. Ingredients of the reaction mixture are listed in Table 3.5. 'Maximo Taq DNA Polymerase' was used like step-1. Thermal cycling protocol is enumerated in Table 3.5. Product of step-1 PCR was used as template for the step-2. Expected final product sizes were 418 bp (*pfmdr1*) and 145 bp (*pfcrt*) respectively.

Table 3.5. Reaction mixture and thermal cycling in step- 02 (nested)

Name of the reagents	Vol. x 1 sample (µL)	Final concentration	Thermal cycling	
10x Standard Taq Buffer	2.5	1 x	step 01	94 °C for 3min
10 mm dNTPs	1.0	0.4 mM	step 02	94 °C for 30 sec
50 mM MgCl2	1.5		step 03	47 °C for 30 sec
M2 Fw	1.5	0.6 µM	step 04	68 °C for 1 min
M2 Rev	1.5	0.6 µM	step 05	go to step 02 (40 cycles)
C2 Fw	3.0	1.2 µM	step 06	68 °C for 3 min
C2 Rev	3.0	1.2 µM	final hold	12 °C
Taq DNA polymerase	0.3			
d. H2O	7.7			
Template DNA (product of step-1)	2.0			
Total	25.0			

3.10.2 Restriction Fragment Length Polymorphism (RFLP) analysis

PCR products were used without any purification procedure for digestions with restriction enzyme. Restriction enzyme Xpal (Apol) of Thermo Fisher Scientific, USA was used in this RFLP. It cleaves the wild types of *Pfcrt* allele in 76K position and *Pfmdr1* in 86N position.

Procedure

- The mixture

PCR product	10 µL
Nuclease free water	18 µL
10x buffer Tango	2 µL
Restriction enzyme Xpal	1 µL

- Mixed gently and spined down for few seconds.
- Incubated at 37° C for about 12 hours.

3.10.3 Gel electrophoresis

The products of multiplex nested PCR, after digestion with restriction enzyme were analyzed by electrophoresis on a 2% agarose gel. The cleaved fragments of *Pfcrt* and *Pfmdr1* were categorized by their molecular weight.

3.11 Genotyping of *pfk13* gene of *P. falciparum*

Mutations in K 13 propeller gene (*pfk13*) of *P. falciparum* have been identified to be associated with artemisinin resistance (Ariey *et al*. 2014). Out of 200 nonsynonymous mutations in the K13 gene reported so far, 9 are validated and more 11 are identified as candidates or associated with artemisinin resistance as mentioned by WHO in 'Artemisinin resistance Global Status Report' in August 2018(WHO,2018[c]).

3.11.1 PCR assay

The k13 propeller region of *P. falciparum* DNA was amplified by a nested PCR following the procedures developed by Didier Ménard and Nimol Khim, Paseur Institute, Cambodia and Paris, France in 2013 (Menard *et al.* 2013).

Primers

Two pairs of primers were used in 2 steps of PCR assays (Table 3.6). Primers were synthesized at Bioneer Corporation, Republic of Korea.

Table 3.6. Sequence of the primers used for step-1 and step-2 (nested) PCR.

Primer name	**PCR**	**Sequence (5' – 3')**
K13_PCR_F	Step-1 PCR	CGGAGTGACCAAATCTGGGA
K13_PCR_R		GGGAATCTGGTGGTAACAGC
K13_N1_F	Step-2 PCR (nested)	GCCAAGCTGCCATTCATTTG
K13_N1_R		GCCTTGTTGAAAGAAGCAGA

Step-1 PCR

Each of the reactions was performed in a final volume of 25 µl mixture. Calculations of the ingredients are presented in Table 3.7. Taq DNA Polymerase used in these reactions was from Invitrogen, Thermo Fisher Scientific, USA. Purified *pf* DNA was used as template. Steps of thermal cycling are enumerated in Table 3.8.

Table 3.7. Master Mix calculation for step-1 PCR

Reagents	Stock Conc.	Final Conc.	Vol. X 1 sample
Nuclease-free water	-	-	11.00 µl
10X PCR Buffer	10X	1X	2.50 µl
$MgCl_2$	25 mM	2.5 mM	2.50 µl
dNTP (each)	2 mM	0.2 mM	2.50 µl
Primer - K13_PCR_F	10 µM	0.25 µM	0.625 µl
Primer - K13_PCR_R	10 µM	0.25 µM	0.625 µl
Taq Polymerase	5U/µL	1.25 U	0.25 µl
Template DNA			5.00 µl
Total			25 µl

Table 3.8. Thermal cycling in step-1

Step no.	Cycle	Temperature (°C)	Time (min)	No. of cycles
1.	Initial Denaturation	95	15:00	1
2.	Denaturation	95	0:30	
3.	Annealing	58	2:00	30
4.	Extension	72	2:00	
5.	Final extension	72	10:00	1

Step-2 PCR (nested)

The volume of each reaction mixture in step-2 PCR was 50 µl. Amplicon of the step-1 PCR was used as template. Ingrdients are shown in Table 3.9. Invitrogen Taq DNA Polymerase' was used like step-1. Thermal cycling protocol is shown in table 3.10. Expected final product size was 849 bp (*pfk13* gene).

Table 3.9 Master Mix calculation for step-2 (nested) PCR

Reagent	Stock Conc.	Final Conc.	Vol. X 1 sample
Nuclease-free water			27.10 µl
10X PCR Buffer	10X	1X	5.00 µl
$MgCl_2$	25 mM	2.5 mM	5.00 µl
dNTP (each)	2 mM	0.2 mM	5.00 µl
Primer - K13_N1_F	10 µM	0.25 µM	1.25 µl
Primer - K13_N1_R	10 µM	0.25 µM	1.25 µl
Taq Polymerase	5U/µL	2.0 U	0.40 µl
Template DNA			5.00 µl
Total			50 µl

Table 3.10. Thermal cycling in step-2

Step no.	Cycle	Temperature (ºC)	Time (min)	No. of cycles
1.	Initial Denaturation	95	15:00	1
2.	Denaturation	95	0:30	
3.	Annealing	60	1:00	40
4.	Extension	72	1:00	
5.	Final extension	72	10:00	1

3.11.2 Gel electrophoresis

The products of this nested PCR were analyzed by electrophoresis on a 2% agarose gel stained with ethidium bromide and visualized under UV trans-illumination. Size of PCR products were estimated against a 100 bp DNA ladder marker.

3.11.3 Sequencing

Amplified products of the nested PCR were sequenced at icddr,b Dhaka, Bangladesh. The PCR products were purified using the ExoSAP-IT method. Sequencing was carried out in an automated ABI 3500 XL genetic analyzer (Applied Biosystems, Foster City, USA). The consensus forward and reverse primers were used separately to amplify and sequence gene.

3.11.4 Sequence analysis

The BioEdit Sequence Alignment Editor (ver. 7.0.9.0) was used to analyze the sequences. The sequences were aligned against the *P. falciparum* 3D7 strain (PF3D7_1343700, PlasmoDB Release 28) using Clustal Omega programme.

3.12 Data analysis

3.12.1 Molecular data

Molecular data obtained through different techniques and procedures like PCR, RFLP, agarose gel electrophoresis and gene sequencing are compiled, compared with existing '*P. falciparum* drug resistance' data and presented in the results section of this thesis.

3.12.2 Clinical and epidemiological data

IBM SPSS version 19 was used for analysis of data. Before entering into the software the data were checked, cleaned and verified routinely to exclude any error or inconsistency. Incomplete and erroneous data were discarded. The entry range and consistency were checked. Then data were analyzed according to the objectives of the study.

4.0 Results

A total of 252 blood samples in the form of 'dried blood spots on filter paper' were collected from *P. falciparum* malaria confirmed (either by RDT or microscopy) Bangladeshi troops working in CHT, Bangladesh, and five Sub-Saharan African countries. Molecular procedures and techniques like DNA extraction PCR, RFLP, sequencing and ultimately genotyping of particular genes of *P. falciparum* involved in drug resistance were carried out on all positive samples. Molecular data obtained through these procedures are compiled and organized according to the objectives of this study.

Clinical, demographic and epidemiological data, collected through in-person interview of the patients and their clinical records, were organized into different groups of variables. Data collected on 'incidence of malaria under mefloquine prophylaxis'in a Bangladeshi military contingent of 738 men in Liberia were also analyzed according to the objectives of this study. Appropriate descriptive and analytical statistics were attempted in analysis of all data.

4.1 Geographic distribution of blood samples

Out of 252 *P. falciparum* positive blood samples, 94 were collected from Rangamati, Khagrachari, Bandarbans and Ramu of Bangladesh and 158 from five Sub-Saharan African countries (Table 4.1).

Table 4.1. Geographic distribution of blood samples

Origin of sample	Area/country	No of samples	Total
Bangladesh	CHT	94	94
Africa	D R Congo	74	158
	Ivory Coast	44	
	Liberia	12	
	Mali	13	
	C A R	15	
Total			252

4.2 Identification of *P. falciparum* species in the blood samples

P. falciparum species was confirmed in 35 out of 94 blood samples from Bangladesh and 45 out of 158 samples from Africa through a nested based PCR (Table 4.2), which targeted the 18 ssr-RNA gene amplifying a 1.2kb fragment of *pf* DNA. Two genus-specific primers were used for the first cycle of amplification. Product of the first amplification was used as template for second amplification cycle, in which parasite species was confirmed using species specific primers. The expected final band size was 205 base pair. The presence of amplification product was detected by simple ethidium bromide staining following agarose gel electrophoresis (Figure 4.1).

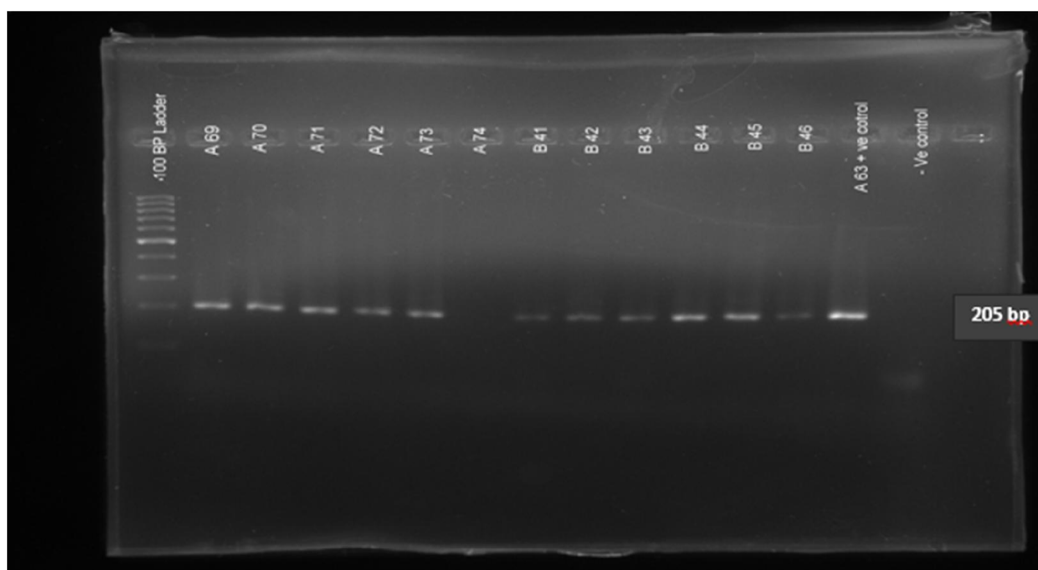

Figure 4.1. Agarose gel electrophoresis of nested PCR products: Bangladeshi and African samples were coded with the alphabets B and A respectively**.** Sample A74 and the negative control did not show any band, rest of the samples including the positive control (A63) showed band of 205 bp measured against a 100 bp DNA ladder.

Table 4.2. Summary of *P. falciparum* species identification

Origin of sample	No of sample	Confirmation of *pf* species by nested PCR	Percentage
Bangladesh	94	35	37.23%
Africa	158	45	28.48%
Total	252	80	31.74%

Proportion of PCR confirmed cases among the patients diagnosed by microscopy and ICT/RDT

Patients included in this study were labeled as cases of falciparum malaria by the treating health facilities through microscopy or ICT/RDT. We found that, 43% microscopically diagnosed patient were confirmed to have *P. falciparum* by PCR, whereas only 28% of ICT/RDT diagnosed cases were confirmed by PCR. (Fig. 4.2).

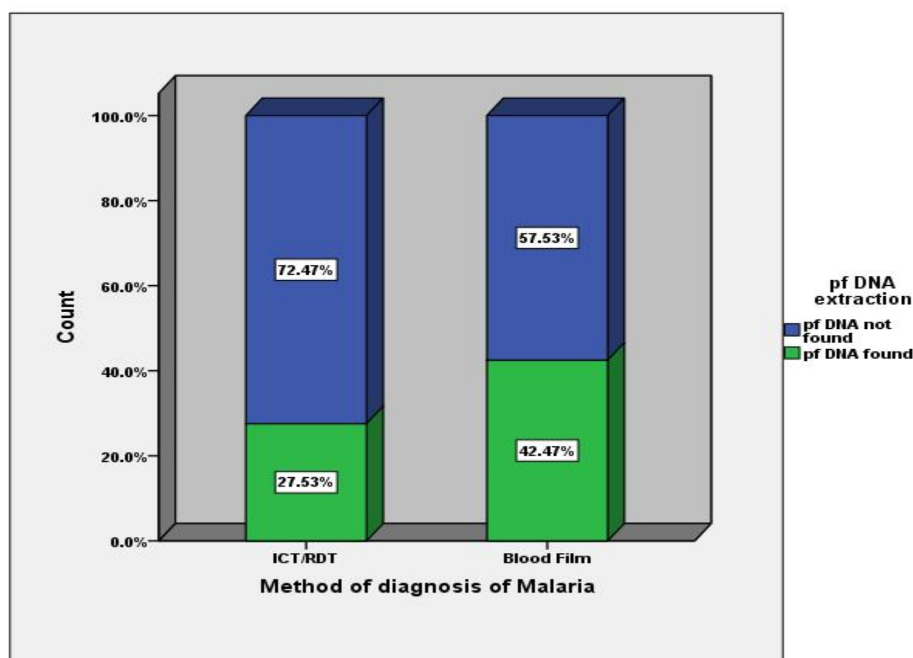

Figure 4.2 Proportion of PCR confirmed cases among the patients diagnosed by microscopy and ICT/RDT

4.3 Genotyping of *pfmdr1* and *pfcrt* genes of *P. falciparum* for drug resistance status

A multiplex nested PCR followed by RFLP was employed for genotyping *pfcrt* and *pfmdr1* genes among the samples confirmed to have *pf* DNA by previous diagnostic PCR. Two nest primers pairs were used simultaneously, amplifying two fragments, one fragment of *pfmdr1* and the other a *pfcrt* amplicon. The amplicons resulted in sizes well distinguish in 2% agarose gel, 418 and 145 bp, respectively. Digestion with ApoI of the '86N harboring *pfmdr1* specific PCR product' gives rise into a 239 and 179 bp fragments. The restriction of the 76K carrying *pfcrt* PCR amplicon results in a 98 and 47 bp fragment (Figure 4.3 and Figure 4.4).

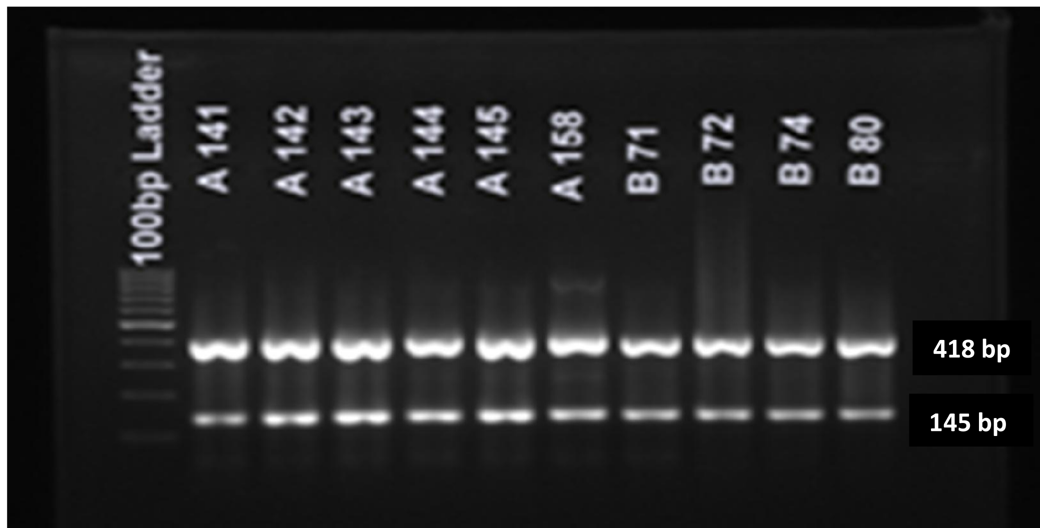

Figure 4.3. Agarose gel electrophoresis of the products of multiplex nested PCR *pfmdr1* gene and *pfcrt* gene fragments of 6 African (A141, A142, A143, A144, A145 and A158) and 4 Bangladesi (B71, B72, B74 and B80) isolates are seen at 418 bp and 145 bp respectively against a 100 bp DNA ladder.

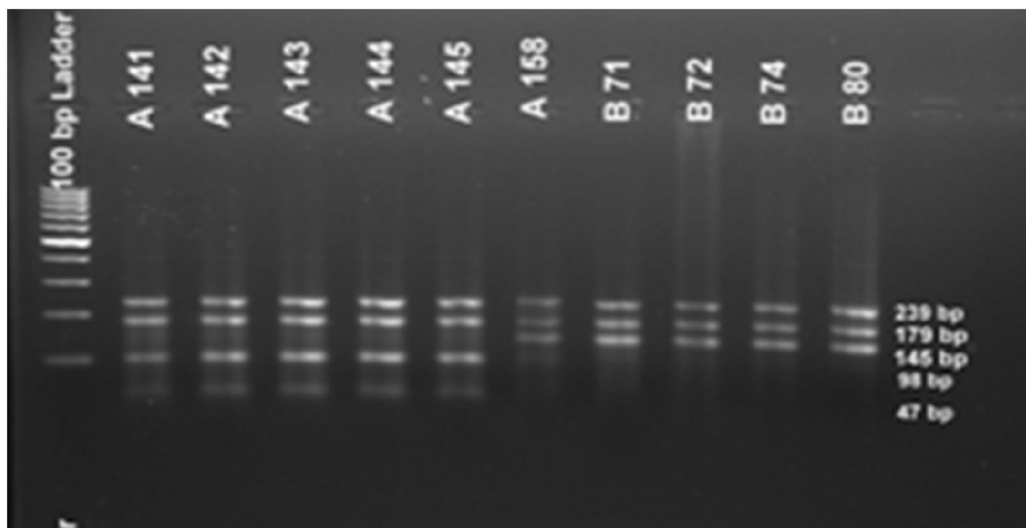

Figure 4.4. Gel run of the amplicons after digestion with restriction enzyme Amplicons of *pfmdr1* (carrying 86N), cleaved into 239 bp and 179 bp fragment after digestion with restriction enzyme, denote wild type allele. Likewise, amplicons of *pfcrt* (carrying 76K), cleaved into 98 bp and 47 bp fragments, denote wild type allele. Whereas, amplicons of both the genes, not cleaved at all, denote mutant type allele**.** The positive and negative controls are not shown in this gels as they were run in another gel with few other samples.

4.3.1 Results of genotyping of *pfmdr1 and pfcrt* genes

Almost 21% *pfmdr1* allele from Bangladesh were mutant, while in Africa, only 2.44% *pfmdr1* allele were found to be mutant denoting resistance to mefloquine and lumifantrine (Table 4.3). In case of *pfcrt* gene, 93% Bangladeshi allele and 29% African allele were mutant denoting resistance to chloroquine (Table 4.4).

Table 4.3. Summary of genotyping of *pfmdr1* gene by PCR-RFLP method

Origin of isolates	Molecular confirmation of *Pf* species	Genotyping of *pfmdr1* by PCR-RFLP method		
		***pfmdr1* amplified**	**Wild**	**Mutant (N86Y)**
Bangladesh	35	29	23 (79.31%)	06 (20.69%)
Africa	45	41	40 (97.56%)	01(02.44%)
Total	80	70	63 (90.00%)	07 (10.00%)

Table 4.4. Summary of genotyping of *pfcrt* gene by PCR-RFLP method

Origin of isolates	Molecular confirmation of *Pf* species	Genotyping of *pfcrt* by PCR-RFLP method		
		***pfcrt* amplified**	**Wild**	**Mutant (K76T)**
Bangladesh	35	29	02 (6.90%)	27 (93.10%)
Africa	45	41	29 (70.73%)	12 (29.27%)
Total	80	70	31 (44.29%)	39 (55.71%)

4.3.2 Country wise comparison of mutation in *pfmdr1* and *pfcrt* genes

Country wise data shows that *pfmdr1* N86Y mutation was present in 21% and 44% isolates from Bangladesh and Congo respectively. On the other hand, *pfcrt* K76T mutation was present in 93%, 24% and 44% isolates from Bangladesh Congo and Mali respectively. None of the isolates from Congo had *pfmdr1* N86Y mutation and none of the isolates from Ivory Coast had either of the mutations. (Fig. 4.5 and 4.6).

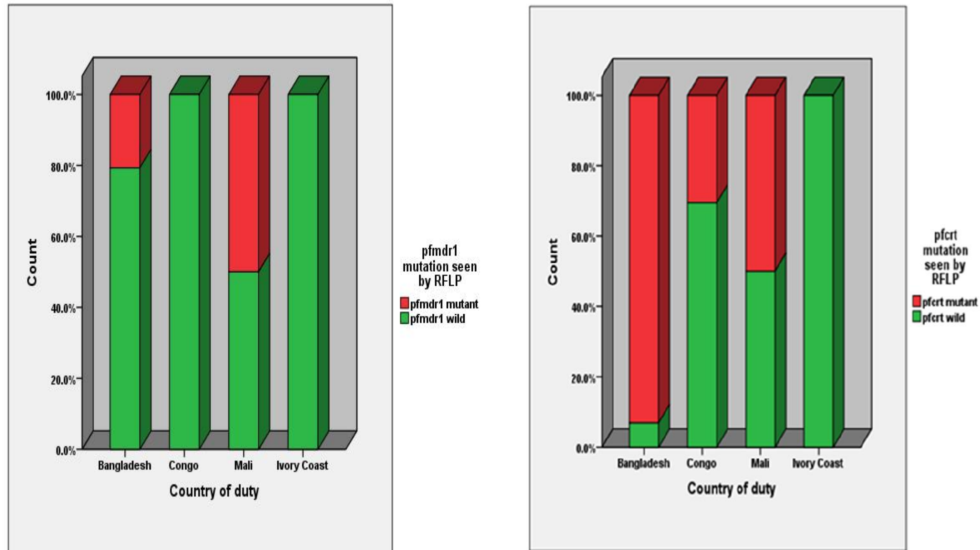

Figure 4.5. Country wise picture of mutation *in pfmdr1* and *pfcrt* gnes

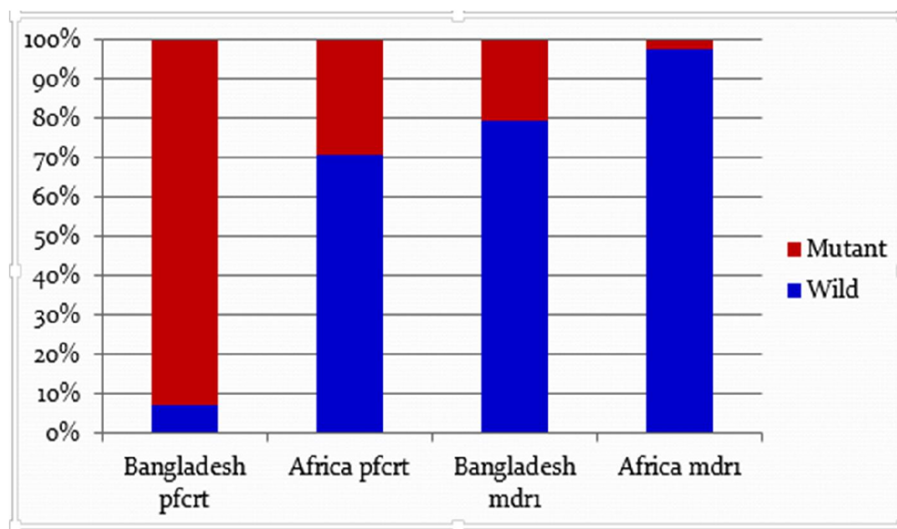

Figure 4.6. Comparison of presence of mutation in samples from Bangladesh and Africa

4.4 Genotyping of *pfk13* gene

Amplification of k13 propeller region of *P. falciparum* was successfully done by a nested PCR (as described by Menard *et al.*2013), in 70 out of 80 PCR positive *pf* isolates from Bangladesh and Africa. Sequencing of *pfk13* gene was done in 69 isolates. The amplified products were analyzed by electrophoresis on an ethidium bromide stained 2% agarose gel. Size of PCR products were estimated against a 100 bp DNA ladder marker (Figure 4.7).

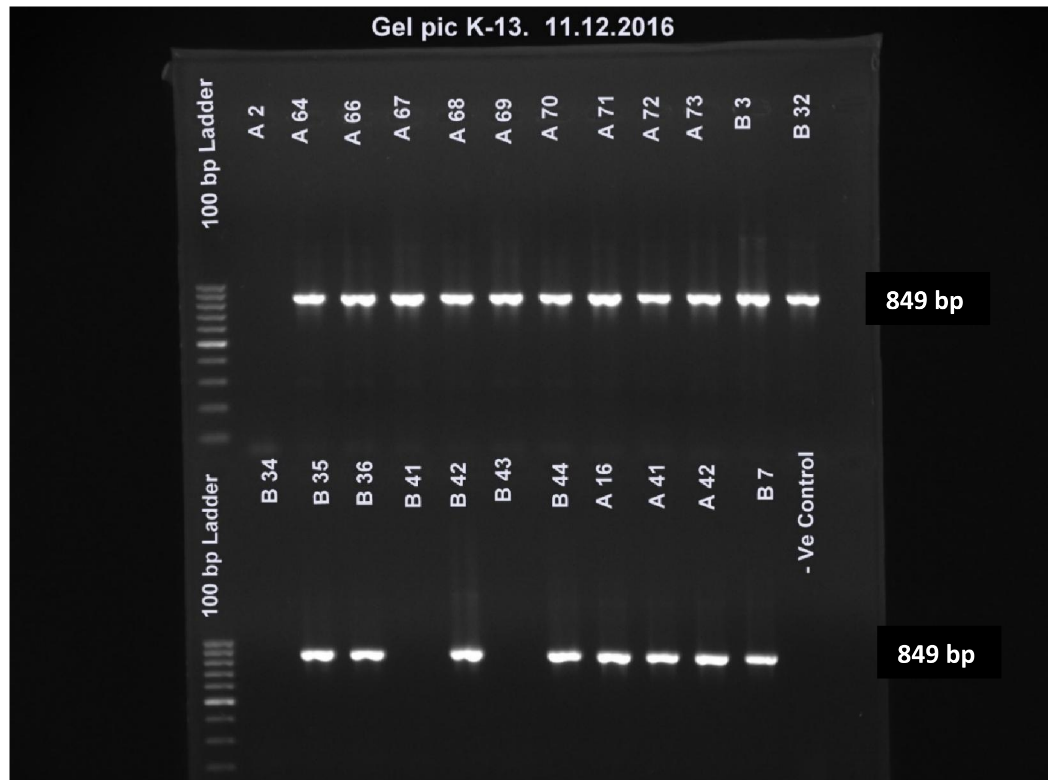

Fig 4.7. Agarose Gel run of nested PCR product of *pfk13* Amplicons of *pfk13* are visualized at the level of 849 bp against a 100 bp DNA ladder. As seen in this gel, amplification was successful in 13 African and 8 Bangladeshi isolates. Bangladeshi isolates B41 and B43 failed to amplify. Sample B7 was run as a positive control as it was confirmed to be *pf* positive by PCR at another laboratory (ICDDR,B).

4.4.1 Results of sequencing of *pfk13*

Sequencing was performed with amplified products of *pfk13* of 40 African and 29 Bangladeshi isolates using the Sanger sequencing method on an ABI3500 Genetic Analyzer. Sequence analysis was done using BioEdit Sequence Alignment Editor. The sequences were aligned against the *P. falciparum* 3D7 strain (PF3D7_1343700, PlasmoDB Release 28) using Clustal Omega programme.

K13 propeller mutations were observed in 9 out of 69 samples underwent sequencing. All 29 Bangladeshi isolates were found to be wild. Nine (22.50%) out of 40 African isolates were mutant, rest 31 (77.50%) were found to be wild (Table 4.5).

Table 4.5. Summary of the results of sequence analysis

Origin of isolates	No of sequencing performed	Results	
		Wild	Mutant
Africa	40	31 (77.50%)	9 (22.50%)
Bangladesh	29	29 (100%)	0 (0.00%)
Total	69	60 (86.96%)	9 (13.04%)

4.4.2 Mutations in *pfk13* gene

This study identified 10 SNPs leading to 6 non-synonymous and 3 synonymous mutations in k13 propeller region in 9 African samples. The S491F mutation was detected in two samples from D R Congo. Eight out of these 9 mutations are being reported herewith for the first time and are identified in samples from D.R.Congo. The other mutation, A578S, identified in a sample from Ivory Coast, is the most frequent mutation observed in Africa so far, although it has not been associated with clinical or *in vitro* resistance to artemisinin. However, 7 out of these 10 mutations are non-synonymous and three mutations are synonymous (Table 4.6).

Table 4.6. Mutations in *pfk13* gene

Serial	Sample code	Country of origin	Mutations	Mutation type	Remarks
1	A64	Aveba, Ituri, Congo	F614F, A617P	Synonymous non-synonymous	New New
2	A67	Aveba, Ituri, Congo	Y616F	Non-synonymous	New
3	A70	Aveba, Ituri, Congo	S491F	Non-synonymous	New
4	A71	Aveba, Ituri, Congo	S491F	Non-synonymous	New
5	A76	Aveba, Ituri, Congo	I616I	Synonymous	New
6	A79	Aveba, Ituri, Congo	N458k	Non-synonymous	New
7	A81	Aveba, Ituri, Congo	K503K	Synonymous	New
8	A145	Bukiringi, Ituri, Congo	Y616F	Non-synonymous	New
9	A131	Man, Ivory Coast	A578S	Non-synonymous	Most common African mutation

4.4.3 Relation between presence of *Pfk13* mutation and duration of fever

K13 propeller region of *P. falciparum* DNA was sequenced in 69 isolates from Bangladesh and Africa. We tried to find out whether there was any statistical relation existed between presence of *Pfk13* mutation and duration of fever the patients suffered. However, χ^2 test revealed no statistical relation between those two variables ($p > 0.05$) (Table 4.7).

Table 4.7. Distribution of the respondents by duration of fever in response to presence of mutation in *Pfk13* gene

Presence of mutation in *Pfk13* gene	Duration of fever (N=69)			
	1 - 4 days	5 – 7 days	≥ 8 days	Statistics
Pfk13 wild allele	27 (45.0%)	25 (41.7%)	8 (13.3%)	χ^2 =2.34 P=0.31
Pfk13 mutant allele	3 (33.3%)	3 (33.3%)	3 (33.3%)	
Total	30 (43.5%)	28 (40.6%)	11 (15.9%)	

4.4.4 Presence of *pfk13* mutation among *pfcrt* and *pfmdr1* wild and mutant isolates

Almost 15% of the isolates having wild type *pfmdr1*gene have k13 propeller mutation. Whereas, none of the isolates having mutant type *pfmdr1*gene have k13 propeller mutation (Figure 4.8). On the other hand, 20% of the wild and 7% of the mutant isolates of *pfcrt* have k13 propeller mutation (Figure 4.9). However, statistical test Chi-square could not reveal any statistical relation between presence of mutations in *pfk13* gene and *pfcrt* and *pfmdr1* gene among the isolates.

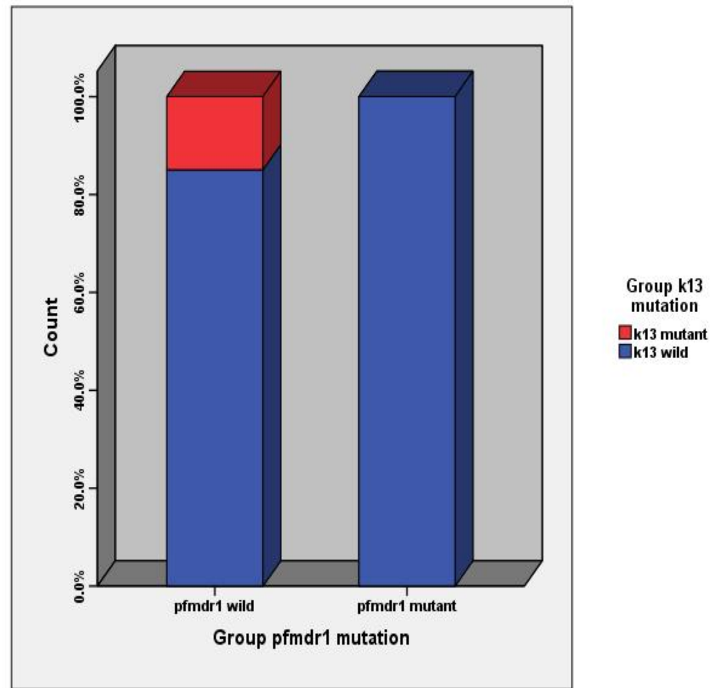

Figure 4.8. Presence of k13 propeller mutation in *pfmdr1* wild and mutant isolates

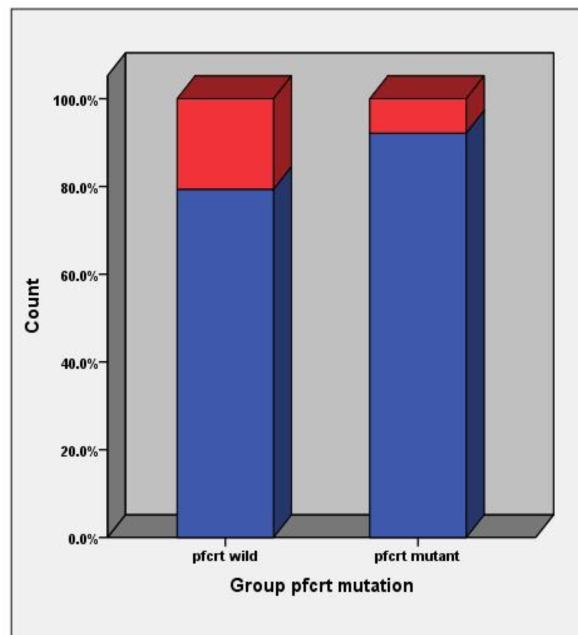

Figure 4.9. Presence of k13 propeller mutation in *pfcrt* wild and mutant isolates

4.5 Some demographic features of the respondents

4.5.1 Age

Highest proportion (50.79%) of the respondents were from the age group 21-30 years followed by 36.90% from 31-40 years, 9.52% from 41-50 years and 2.78% from the age group 20 years and bellow (Figure 4.10).

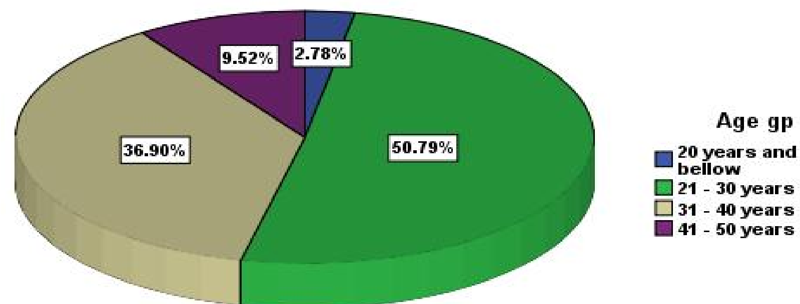

Figure 4.10. Age group of the respondent troops

4.5.2 Type of force

Bangladesh Army constituted the major proportion (83.73%) of the respondents. Rest of the respondents were from Border Guards of Bangladesh, Bangladesh Ansar, Bangladesh Police and other forces of Bangladesh. This study was restricted within Bangladeshi nationals because of ethical issues (Figure 4.11).

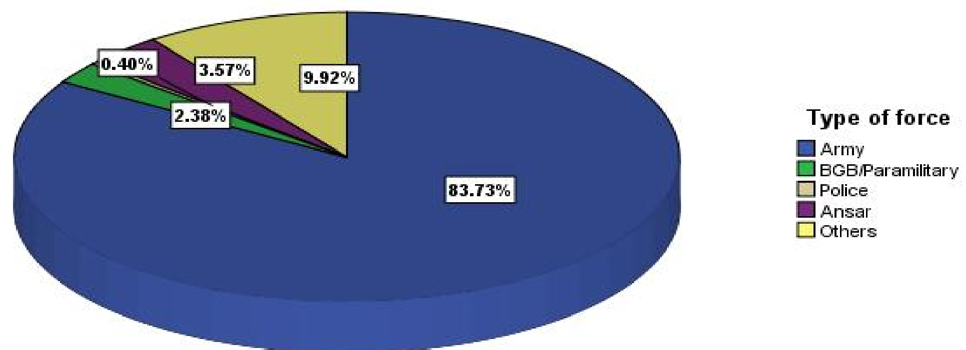

Figure 4.11. Organizational affiliation of the respondent soldiers

4.5.3 Nature of job

Almost three fourth (72.62%) of the respondents were occupied in general military duty like patrolling, security duty etc. Rest of them office work, cleaning, driving and other jobs (Figure 4.12).

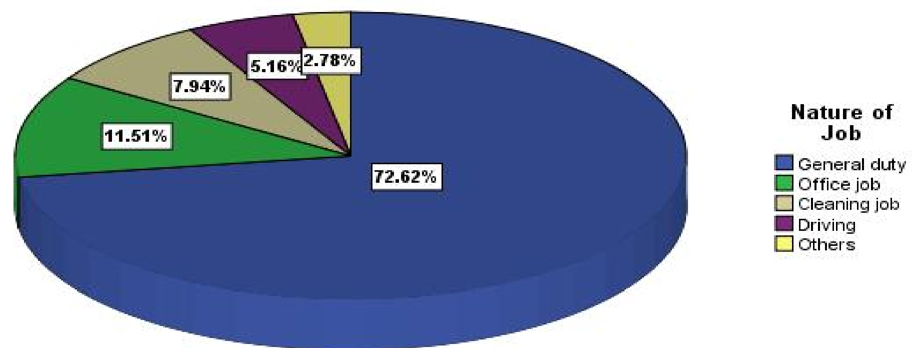

Figure 4.12. Nature of job of the respondent troops

4.6 Analysis of clinical data

4.6.1 1st line antimalarial drug and clinical outcome

A WHO recommended oral combination of artemether and lumifantrine was used as the 1st line antimalarial drug for most of the falciparum malaria cases among Bangladeshi troops in both Bangladesh and Africa. However some of them were treated with other drugs like quinine, injection artemether, chloroquine etc. Patients were followed up for few clinical outcome like duration of fever, number of fever episodes, failure of drug, referral, length of hospital stay etc. Table 4.8, Table 4.9, Table 4.10 and Table 4.11 display distribution of the respondent soldiers by variables namely duration of fever, number of fever episodes, drug failure and referral to higher level hospitals in response to the 1st line antimalarial drug used for their treatment. Statistical test reveals that all of these outcome variables are associated with the 1st line antimalarial drug used ($p < 0.05$).

Table 4.8. Distribution of the respondents by duration of fever in response to 1st line antimalarial drugs

1st line antimalarial drugs	Duration of fever (N=248)			
	1 - 4 days	5 – 7 days	≥ 8 days	Statistics
ACT	79	40	7	χ^2 =45.25 P=0.00
Tab./Inj. Quinine	30	63	22	
Tab. Chloroquine	0	0	1	
Inj. Artemether	1	0	0	
Others	4	1	0	
Total	114	104	30	

Table 4.9. Distribution of the respondents by number of fever episodes in response to 1st line antimalarial drugs

1st line antimalarial drugs	Number of fever episodes(N=248)			
	1-10 times	11-20 times	≥ 21 times	Statistics
ACT	87	38	1	χ^2 =44.15 P=0.00
Tab./Inj. Quinine	60	49	6	
Tab. Chloroquine	0	0	1	
Inj. Artemether	0	1	0	
Others	5	0	0	
Total	152	88	8	

Table 4.10. Distribution of the respondents by drug failure in response to 1st line antimalarial drugs

1st line antimalarial drugs	Drug failure (N=240)		
	Yes	No	Statistics
ACT *	0	123	χ^2 =26.30 P=0.00
Tab./Inj. Quinine	21	91	
Tab. Chloroquine	0	1	
Inj. Artemether	0	1	
Others	0	3	
Total	21	219	

Table 4.11. Distribution of the respondents by referral to higher level hospitals in response to 1st line antimalarial drugs

1st line antimalarial drugs	Referral (N=246)				
	Not referred	To level-I hosp	To level-II hosp	To level-III hosp	Statistics
ACT *	63	47	11	4	χ^2 =23.36 P=0.025
Tab./Inj. Quinine	31	63	14	6	
Tab. Chloroquine	1	0	0	0	
Inj. Artemether	1	0	0	0	
Others	3	0	2	0	
Total	99	110	27	10	

4.6.2 Prophylactic drug and clinical outcome

Bangladeshi troops working in endemic African countries used to take weekly dose of 750 mg mefloquine as anti-malarial prophylaxis, though this practice is not in practice in Chittagong Hill Tracts. Malaria patients under prophylactic mefloquine (in Africa) and those not under prophylactic mefloquine (in Bangladesh) were followed on outcome variables like duration of fever, number of fever episodes, drug failure and mutation in *pfmdr1*(Table 4.12). In statistical analysis only 'number of fever episodes' was fond to be associated with prophylactic mefloquine (p = 0.00).

Table 4.12. Distribution of the respondents by duration of fever and number of fever episodes in response to prophylactic mefloquine

Use of prophylactic mefloquine	Duration of fever (N=252)				Number of fever episodes(N=252)			
	1 - 4 days	5 – 7 days	≥ 8 days	Statis-tics	1-10 times	11-20 times	≥ 21 times	Statis-tics
No prophylactic drug	39	43	12	χ^2 =1.48 P=0.48	40	47	7	χ^2 =25.64 P=0.00
Under prophylactic mefloquine	78	62	18		114	43	1	
Total	117	105	30		154	90	8	

4.7 Incidence of malaria under mefloquine prophylaxis

Within purview of this research work, data was analyzed of a Bangladeshi military contingent of 738 men deployed in Liberia and monitored for 1 year (June 2012 - May 2013) for 'incidence of malaria under mefloquine prophylaxis. The incidence of malaria was 133 (180 per thousand in 1 year). *P. falciparum* was confirmed by ICT/microscopy in 44% of the cases. *P. Falciparum* species was found in all 60 microscopy /RDT confirmed cases. Only 2 cases were found to have mixed infections with *P. falciparum* and *P. vivax* both.

4.7.1 Monthly state of malaria in Bangladeshi military contingent in Liberia

Monthly state of the year (June 2012 - May 2013) shows 2 peaks in the curve, an April peak and a September peak. November to February and May to July may be considered as low incidence seasons (Figure 4.13).

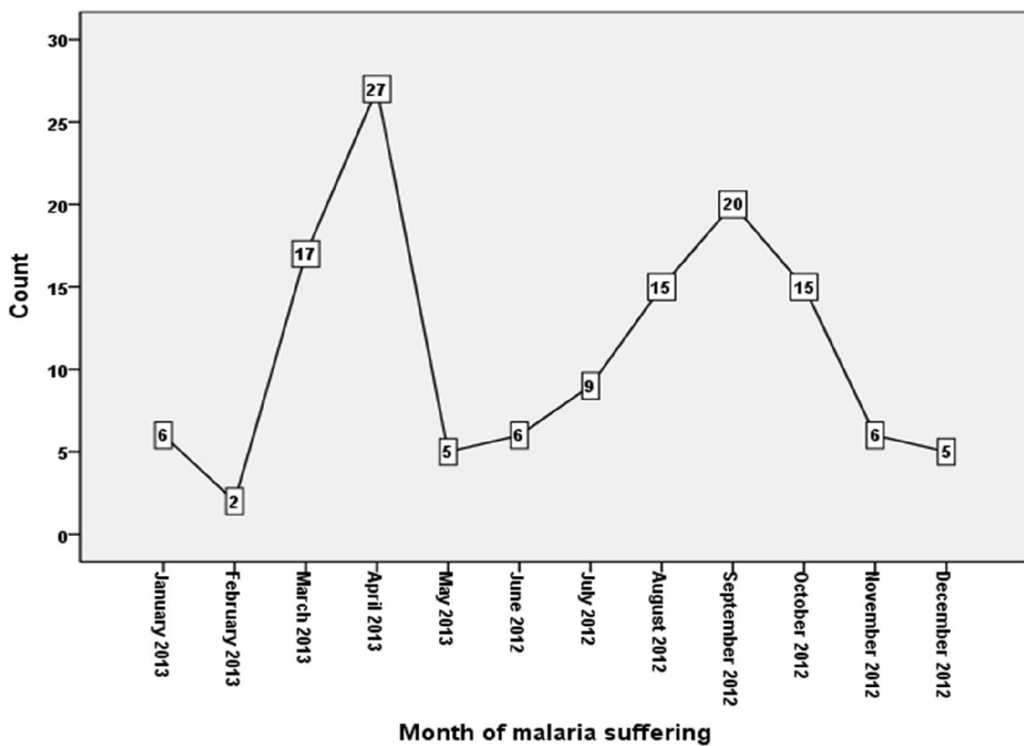

Figure 4.13. Curve showing monthly state of malaria in in Bangladeshi troops in Liberia (Incidence of malaria was studied from June 2012 to May 3013).

4.7.2 Repeated malaria

Among 133 malaria patients in Bangladeshi troops in Liberia 7.52% had malaria repeated for once and 2.26% had malaria repeated for twice (Figure 4.14).

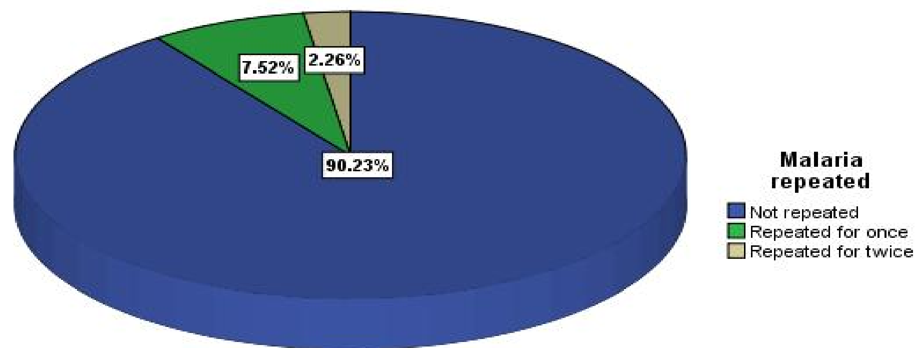

Figure 4.14. State of repeated malaria in Liberia in 1 year

4.7.3 Relationship among different variables

Statistical tests were carried out to find out relationship between different variables used in data collection. Length of febrile period was found to be associated with parasite count (P=0.04), whereas no association was detected between febrile period and time spent before starting antimalarial drug and also between range of temperature and parasite count (p>0.05), as shown in Table 4.13, Table 4.14 and Table 4.15.

Table 4.13. Distribution of malaria patients by length of febrile period according to the time spent before starting anti-malarial drug from the onset of fever

Time spent before starting anti-malarial from the onset of fever	Length of febrile period in days			Statictics
	≤ 2 days	**3-5 days**	**≥ 6 days**	
1 hour	65	14	2	
2-3 hours	19	6	0	
4-5 hours	3	2	0	χ^2 =12.76
6-10 hours	4	2	0	P=0.12
11 hours and more	7	9	0	
Total	98	33	2	

Table 4.14. Distribution of malaria patients by length of febrile period in response to their MP count

Malaria parasite count	Length of febrile period in days			Statictics
	≤ 2 days	**3-5 days**	**≥ 6 days**	
MP negative	47	10	0	
Scanty	6	3	1	
Up to 200 per µL of blood	5	6	1	χ^2 =16.43
201-500 per µL of blood	7	5	0	P=0.04
501-1000 per µL of blood	3	3	0	
Total	68	27	2	

Table 4.15. Distribution of malaria patients by range of temperature in response to their MP count.

Malaria parasite count	Range of temperature in ^{0} F				Statictics
	99-100	99-101	99-102	99-103	
MP negative	9	37	10	1	
Scanty	5	2	3	0	
Up to 200 per µL of blood	2	3	6	1	χ^2 =20.82
201-500 per µL of blood	3	5	3	1	P=0.053
501-1000 per µL of blood	0	5	1	0	
Total	19	52	23	3	

4.8 Analysis of data on knowledge attitude and practice (KAP)

4.8.1 Patients' perception about malaria transmission

Almost 92% of the respondent soldiers identified mosquito bite as the route of transmission of malaria to human body while 31% of them think that other insects may also contribute in the process of transmission. Few of them even believe that dirty environment (2.79%) or cough and sneeze may transmit malaria (Figure 4.15).

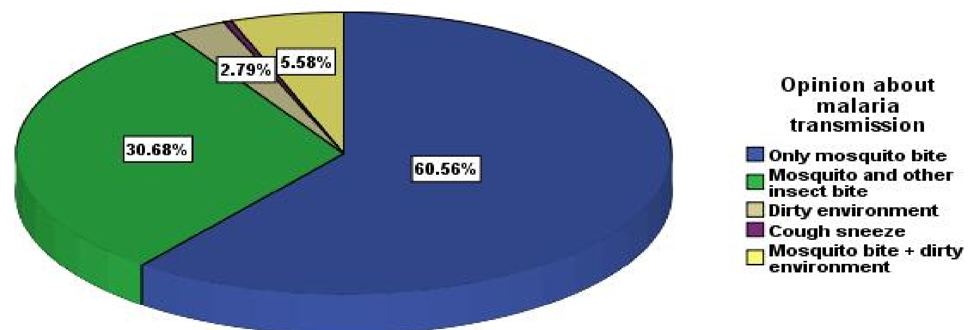

Figure 4.15. Patients' perception about malaria transmission

4.8.2 Patients’ perception about efficacy of prophylactic mefloquine (MQ)

About two third of the respondent soldiers were confident about the efficacy of prophylactic mefloquine,but 2.63% of them think that it does not work at all (Figure 4.16). We compared the proportions of troops confident on mefloquine prophylaxis among two groups, one remaining under this prophylaxis (working in Africa) and the other remaining without this prophylaxis (working in CHT, Bangladesh). Almost 76% of the ‘respondents under MQ prophylaxis’were affirmative about the efficacy of the drug whereas, in the other group only 39.4% respondents were affirmative. The difference is statistically significant (P=0.00) (Table 4.16).

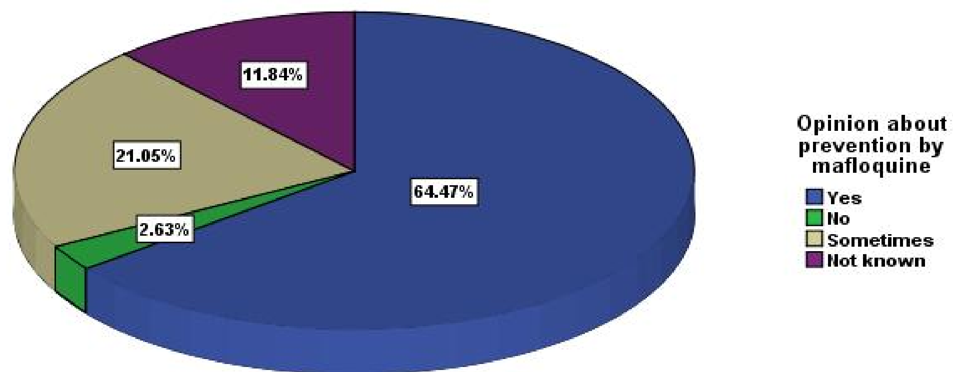

Figure 4.16. Patients’ perception about efficacy of prophylactic mefloquine

Table. 4.16. Distribution of respondents by their opinion on efficacy of MQ prophylaxis

State of MQ prophylaxis	Soldiers' opinion on efficacy of MQ prophylaxis				
	Yes	No	Sometimes	Not known	Statistics
Soldiers not on MQ prophylaxis	28 (39.4%)	3 (4.2%)	14 (19.7%)	26 (36.6%)	χ^2 =64.56 P=0.000
Soldiers on MQ prophylaxis	119 (75.8%)	3 (1.9%)	34 (21.7%)	1 (0.6%)	

4.8.3 Patients' perception about cause of resistance

Thirty two percent of the respondents opined that malaria parasite develops drug resistance naturally, while 24% of them blame low quality medicines for drug resistance. Rest of the respondents were divided into groups with opinions like irregular intake of medications, environmental change and combinations of above mentioned variables (Figure 4.17).

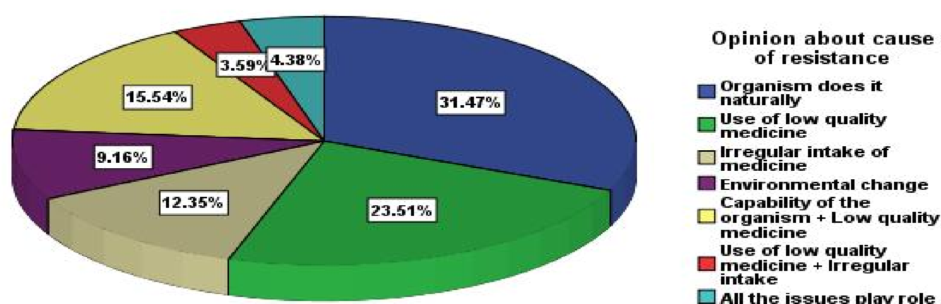

Figure 4.17. Patients' perception about cause of resistance

4.8.4 Patients' perception about priority of preventive options

Almost two third of the respondent soldiers recognized the use of mosquito net as the top priority malaria preventive measure. Rest of the respondents were divided over the opinions of indoor aerosol/coil, outdoor spray, protective clothing etc. as top priority preventive measure (Figure 4.18).

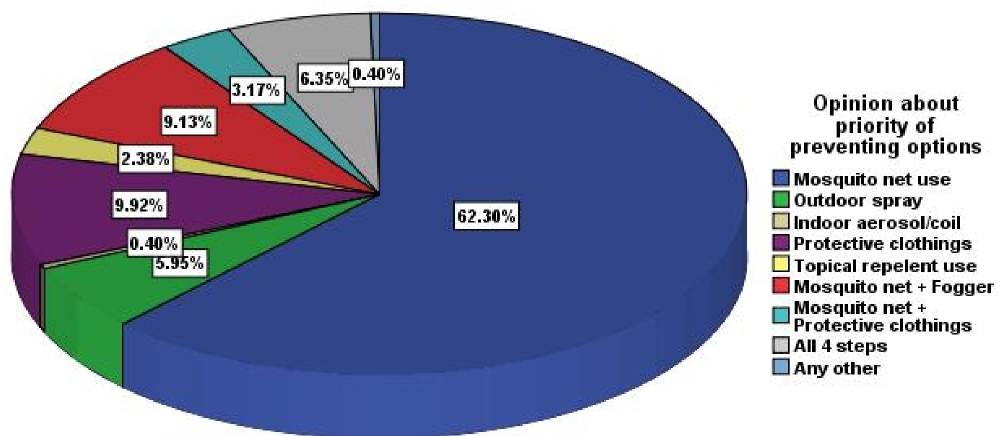

Figure 4.18. Patients' perception about priority of preventive measures

4.8.5 Patients' source of information about malaria

Fifty five percent of the respondents received information about malaria from health personnel. 29.97% of them got such information from their superior authority. Rest of the respondents got such information through self-study and other sources (Figure 4.19).

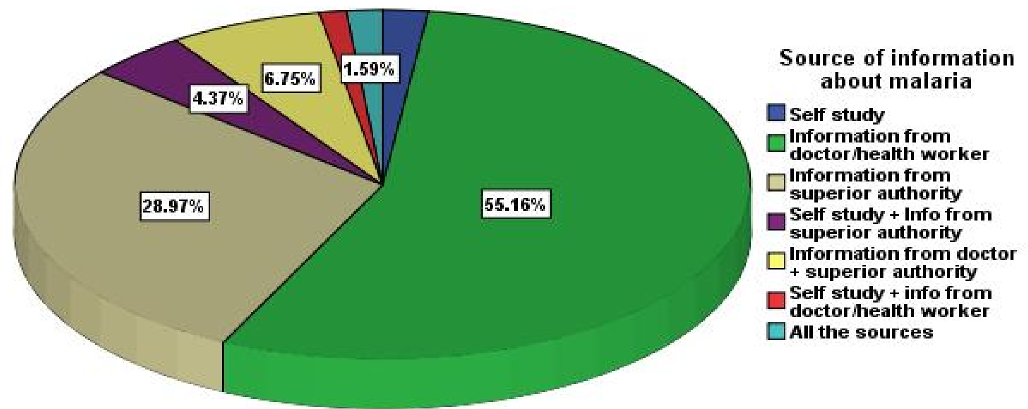

Figure 4.19. Patients' source of information about malaria

4.8.6 Source of information and patients' perceptions

Respondents were dependent on different sources for information about malaria. Statistical analysis shows that, the correctness of their opinion on malaria transmission, cause of resistance and preventive priorities is associated with their source of information ($p < 0.05$) (Table 4.17).

Table 4.17. Distribution of the respondents by their opinion on malaria transmission, cause of resistance and preventive priorities depending on different sources of information

Source Of information	Opinion on malaria transmission (N=251)			Opinion on cause of resistance (N=251)			Opinion on preventive priority (N=252)		
	Correct answer	Incorr-ect answer	Stati-tics	Correct answer	Incorr-ect answer	Statis-tics	Correct answer	Incorr-ect answer	Stati-tics
Health personnel	92	46	χ^2 = 12.92 P= 0.04	131	7	χ^2 = 57.10 P= 0.00	102	37	χ^2 = 34.28 P= 0.00
Superior authority	37	36		61	12		45	28	
Self study	2	3		4	1		3	2	
Self study + superior authority	9	2		11	0		2	9	
Health personnel+ superior authority	7	10		8	9		3	14	
Self study + health personnel	3	0		0	3		1	2	
Other sources	2	2		2	2		1	3	
Total	152	99		217	34		157	95	

4.8.7 Preventive practices

4.8.7.1 Use of mosquito net

Majority of the Bangladeshi troops (60% to 90%) used mosquito net while sleeping day and night in all the countries they worked except in Mali where the maximum number of the troops used that net in the night only (Figure 4.20). The proportions of insecticide treated bed net usage among Bangladeshi troops in Bangladesh and Africa are 40% and 5% respectively (over all 19%) (Figure 4.41).

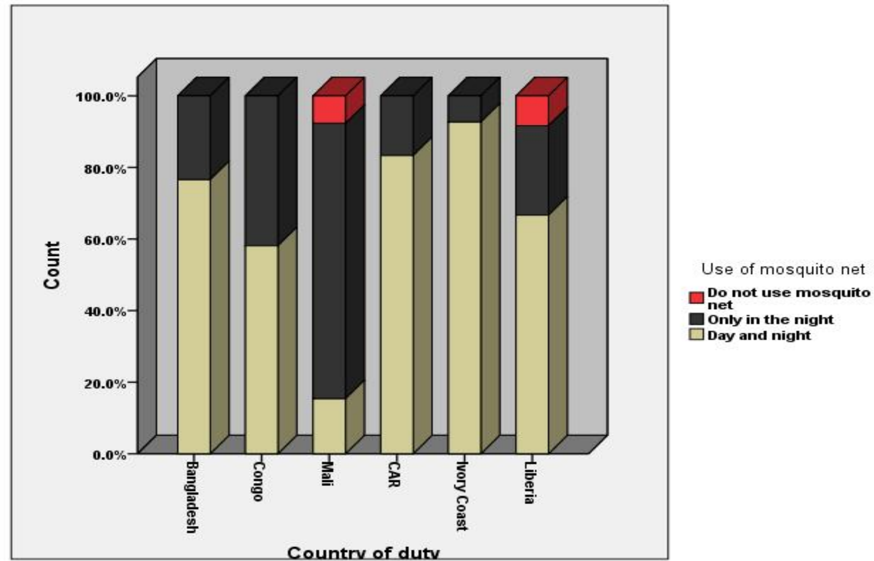

Figure 4.20. Use of mosquito net by the troops working in different countries

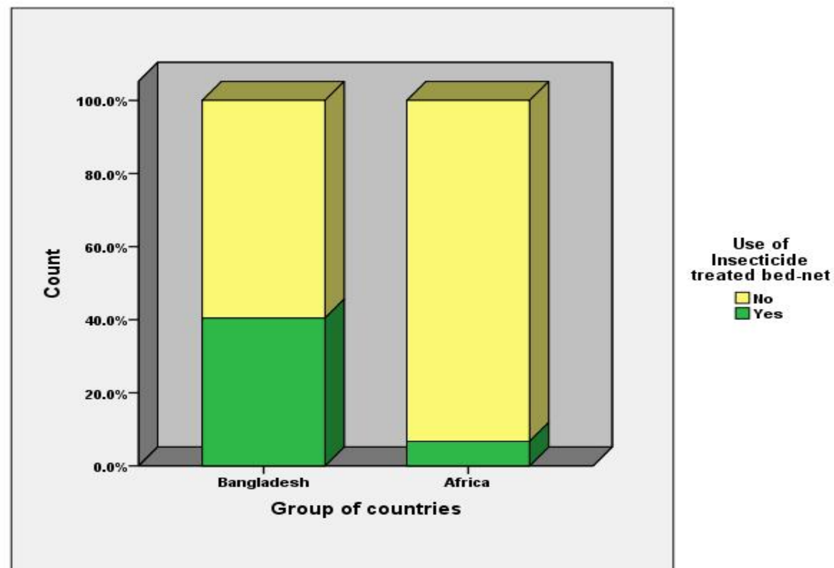

Figure 4.21. Use of insecticide treated bed net by the troops in Bangladesh and Africa

4.8.7.2 Use of protective clothing

Protective clothing like full sleeved shirts, pants and shocks in the evening and early morning were used every day by majority (70%-95%) of the troops working in different countries except in CAR (Figure 4.22).

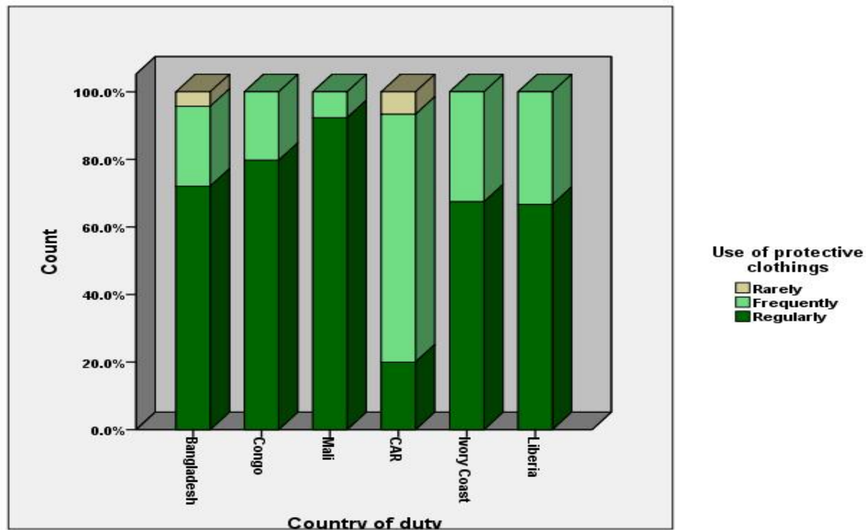

Figure 4.22. Use of protective clothing by troops working in different countries

4.8.7.3 Use of mosquito repellent topical lotion / oil

In endemic countries troops have a practice of using mosquito repellent topical lotion / oil on exposed parts of the body. Troops working in Mali were reluctant to use it every day (Figure 4.23).

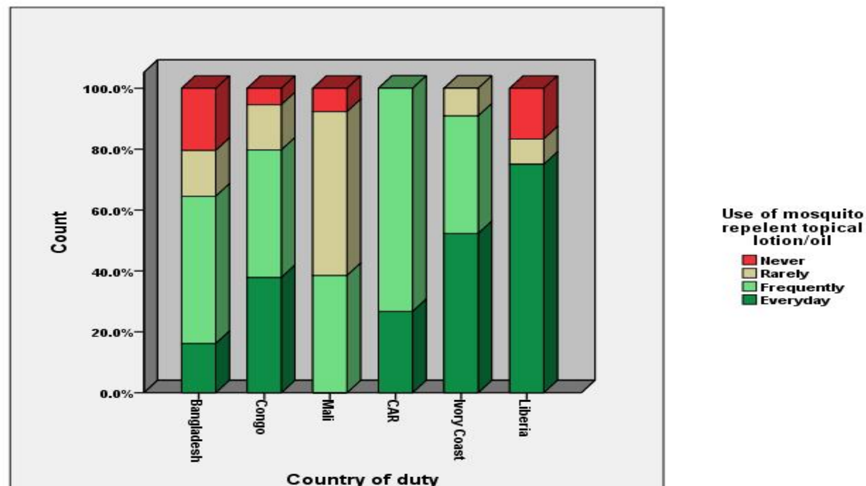

Figure 4.23. Use of mosquito repellent topical lotion/oil by the troops in different countries

4.8.7.4 Use of mosquito repellent indoor spray / coil

Quite a good proportion of the troops in Bangladesh, Congo, Liberia and Mali never used mosquito repellent indoor spray or coil in their tenure. However, some of the troops in all the countries used these repellents frequently (Figure 4.24).

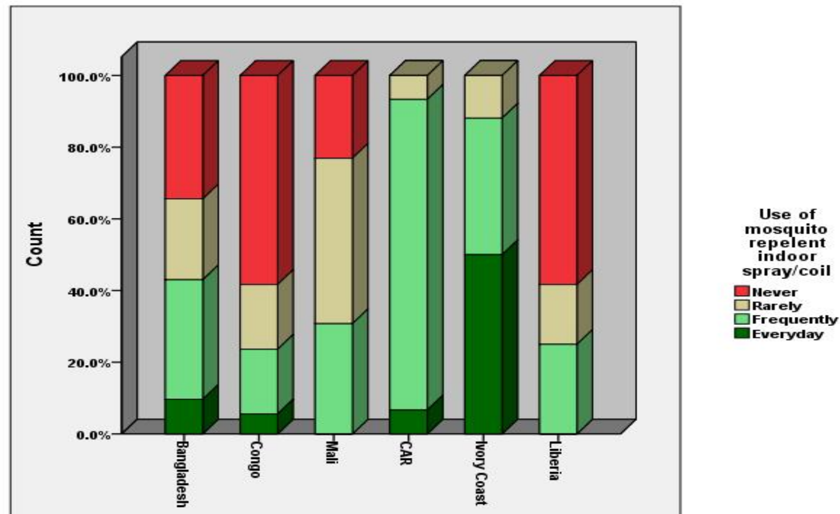

Figure 4.24. Use of mosquito repellent indoor spray / coil by the troops working in different countries

4.8.7.5 Outdoor insecticidal spray

Outdoor mosquito-cidal spray like cypermethrin was arranged by the authority in the camps of Bangladeshi troops in Bangladesh and Africa. In All the countries outdoor spray was observed as a frequent practice (Figure 4.25).

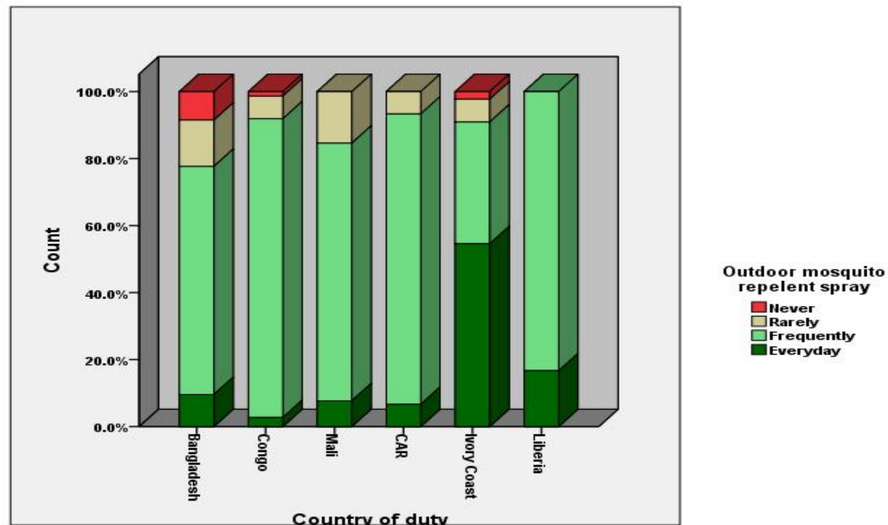

Figure 4.25. Practice of outdoor insecticidal spray by the authority

4.8.7.6 Source of information and preventive behavior

Preventive behavior of the respondent soldiers like use of protective clothing, insecticide treated bed net and mosquito repellent were found to be associated with their source of information ($p < 0.05$) (Table 4.18).

Table 4.18. Distribution of the respondents by their preventive behavior

Source of information	Use of protective clothing (N=250)			Use of insecticide treated bed net (N=243)			Use of mosquito repellent (N=251)		
	Regular	Irregular	Statistics	Yes	No	Statistics	Regular	Irregular	Statistics
Health personnel	110	28	χ^2= 37.10 P= 0.00	18	114	χ^2= 16.47 P= 0.01	103	36	χ^2= 46.82 P= 0.00
Superior authority	41	32		14	57		58	15	
Self-study	3	1		2	3		2	3	
Self-study + Superior authority	11	0		6	5		7	3	
Health personnel + Superior authority	10	7		5	12		14	3	
Self-study + Health personnel	2	1		1	2		3	1	
Other sources	1	3		2	2		2	2	
Total	178	72		48	195		188	63	

5.0 Discussion

The global incidence rate of malaria is in a phase of decline (18% decline between 2010 and 2017). An increasing number of countries are progressing towards elimination (WHO 2018[d]). The situation is almost near to the great eradication programme of last century, which finally failed to the wide spread resistance of malaria parasite to chloroquine, along with few other reasons. The most important challenge to present day elimination progammes is also drug resistance. Drug resistance of *P. falciparum* to multiple anti-malarial drugs and finally development of resistance to artimisinin and its partner drugs are now threatening the achievements of our long fight against malaria.

Armed forces' personnel in South East Asia and Sub-Saharan Africa usually need to operate in malaria endemic areas. Members of Bangladesh Armed Forces operate in endemic areas of both home and Africa because of their deployment at hilly areas at home and peace keeping missions in Africa. Not surprisingly, they need to travel between different endemic regions. Unfortunately, there is no data available on the genetic types of falciparum malaria they suffer or probable resistant strains they might transmit between different endemic areas they travel. The present study is designed to investigate and compare molecular pattern and epidemiological aspects of anti-malarial drug resistance of *P. falciparum* in members of Armed Forces of Bangladesh working in endemic areas at home and Africa.

A total of 252 blood samples in the form of 'dried blood spots on filter paper' were collected from *P. falciparum* malaria confirmed (either by RDT or microscopy) Bangladeshi troops working in CHT, Bangladesh, and five Sub-Saharan African countries. Out of the samples, 94 were collected from Bangladesh and 158 from Africa. African countries include D R Congo, Ivory Coast, Mali, Liberia and Central African Republic.

Extraction of *P. falciparum* DNA was done from all 252 samples and all of them underwent a nested PCR protocol for molecular confirmation of *P. Falciparum.* Thus, 37.23% of Bangladeshi samples and 28.48% of African samples (total 31.74%) were

confirmed to have the particular species of the parasite. Molecular detection rate of *P. falciparum* species among microscopy/RDT conformed blood samples in this study was higher than that of a study conducted by Ogbolu *et al.* (2012) in Nigeria but lower than that of few other studies (Aslan *et al.* 2007, Parajuli *et al.* 2009 and Ahmed R. *et al.* 2018). A lower rate of molecular detection of *P. falciparum* species among microscopy /RDT confirmed dried blood samples in this study might be due to few reasons. Microscopy/RDT were done at the time of sample collection, samples were then preserved usually for months in the field condition before transported long way from CHT/Africa to the laboratory of University of Dhaka. The conflict ravaged field condition and long way transportation through different means might play some role in degradation of DNA in some of the samples. Lack skill of field staffs in microscopy and probable confusion in distinguishing *P. falciparum* from *p. vivax* and other species while doing RDT might also play some role. However, the rate of detection of *pf* DNA in the samples from CHT, Bangladesh was higher than that from African countries.

In this study, 43% microscopically diagnosed patient were confirmed to have *P. falciparum* infection by PCR, whereas only 28% of RDT diagnosed cases could be confirmed by PCR. This result is consistent with the findings of Ojurongbe *et al.* (2013) in a study in Nigeria where sensitivity of microscopy was higher than that of RDT in detecting *P. falciparum*, while in some other studies RDT yielded higher sensitivity over microscopy (Saha *et al.* 2017, Berzosa *et al.* 2018). Owing reverse result in a study, researchers felt it necessary to reinforce training in microscopy (Berzosa *et al.* 2018).

Chloroquine was once the gold standard compound for antimalarial chemotherapy. Development of widespread resistance to this drug was marked with a huge increase in malaria related morbidity and mortality at a global scale in last century. Polymorphism in the *pfcrt* (*P. falciparum chloroquine resistance transporter*) gene, particularly the one amino acid change, K76T, located in the first transmembrane domain, has been found consistently in chloroquine-resistant *P. falciparum* parasites. Another mutation could be at the *pfmd1* (*P. falciparum multi drug resistance 1*) gene encoding for the transporter for importing solutes into the food vacuole, including the

drugs mefloquine and lumifantrine (Boland, 2001). Lumifantrine is a partner drug of artimisinine and constitutes one of the most common and effective ACT of present time. Mefloquine is used as a prophylactic antimalarial drug by travelers including members of Armed Forces of Bangladesh working in Africa.

In the present study, a multiplex nested PCR followed by RFLP method was employed to investigate the presence of *pfcrt* (K76T) and *pfmdr1* (N86Y) mutations in samples from Bangladesh and Africa. Accordingly, *pfcrt* (K76T) mutation was detected in 93.10% Bangladeshi and 29.27% African samples. On the other hand, *pfmdr1* (N86Y) mutation was detected in 20.69% Bangladeshi and only 2.44% African samples.

Molecular evidence of *P. falciparum* resistance to chloroquine and partner drugs happened to be observed much before its removal from National Treatment Guideline as the first-line treatment of uncomplicated *P. falciparum* malaria in Bangladesh in 2004. Ingrid *et al.* (2004) conducted a study in Khagrachari Hill District of Bangladesh in the year 2002, which revealed that, 94% of parasites carried the 76T mutation at *pfcrt*, while 70% carried the 86Y mutation at *pfmdr*. In 2004, Kawai *et al.* (2011) detected *pfcrt* (K76T) mutation in 38 (84%) out of 45 uncomplicated falciparum malaria patients' blood samples from Rangamati Hill District. In an analysis of 33 paired recrudescent infections after drug treatment in the period 2004 to 2008 in the Chittagong Hill Tracts, Akter *et al.* (2012) found prevalence of the *pfcrt* K76T chloroquine resistant allele in approximately 82% of the samples, while the resistant *pfmdr1* (N86Y) allele was present in 33% of the samples.

Although it has been almost a decade since chloroquine had been withdrawn from the National Treatment Guide line, the prevalence of chloroquine resistant *P. falciparum* strains remains high. While carrying out a molecular analysis of 130 *P. falciparum* isolates collected between August 2014 and January 2015 from Bandarban, Alam *et al.* (2017) detected *pfcrt* K76T and *pfmdr1* N86Y mutations in 81.5% and 13.9% isolates respectively. Our present study reveals even higher rates of prevalence of these two mutations (93.10% and 29.27% respectively) in blood samples from Chittagong Hill Tracts of Bangladesh. Fact is that, in Bangladesh, chloroquine

continues to be available in drug stores for self-treatment of malaria and for the treatment of *P. vivax*,that may have maintained chloroquine pressure on the local *P. falciparum* population, explaining the high prevalence of *pfcrt* 76T mutants in this study.

Studies in neighboring countries of Bangladesh reported similar prevalence of mutant genotypes of *P. falciparum* in last couple of years. Pickard *et al.* (2003) reported polymorphism pfcrt Thr76 in 97% of isolates from Thailand, Myanmar, Vietnam, and Bangladesh. Goswami *et al.* (2014) recorded *Pfcrt* K76T and *pfmdr1* N86Y mutations in 100% and 52.6% treatment failure cases in Indo-Bangladesh border areas. High prevalence of CQ and MQ-resistant *P. falciparum* isolates was also observed along the areas of Thai-Cambodian and Thai-Myanmar borders between 2006 and 2009 (Phompradit *et al.* 2014). In samples collected from Chhattisgarh, India between 2013 and 2015, mutation was detected in 78% and 59% *pfcr* and *pfmdr1* gene respectively (Patel *et al.* 2017).

On the other hand prevalence of *pfcrt* (K76T) and *pfmdr1* (N86Y) mutations in African isolates is fairly low in this study (29.27% and 2.44% respectively). Individually, isolates from Congo and Mali had mutations in *pfcrt* gene at rates of 24% and 44% respectively. Isolates from Congo had no mutation in *pfmdr1*gene and isolates from Ivory Coast had no mutation in either of the genes.

In a molecular survey in Congo in 2014 Mvumbi *et al.* (2017) got mutations in *pfcrt* and *pfmdr1* genes among 63.9% and 1.07% samples respectively. Back in 2010 in Congo Koukouikila-Koussounda *et al.* (2012) got mutation in *pfcrt* gene in 92% samples under study. These findings followed by the finding of our study indicate a gradual reduction in the prevalence of mutation in *pfcrt* gene in D R Congo. This may be a consequence of cessation of malaria treatment with chloroquine and introduction of ACTs as 1st line of treatment in 2005.

In Malawi (the 1st African country to replace CQ with sulfadoxine pyrimethamin in 1993), Kublin *et al.* (2003) found that chloroquine-resistant *pfcrt* genotype decreased from 85% in 1992 to 13% in 2000. In West African country of Niger, 6 Years after

withdrawal of CQ and introduction of artemisinin-based combinations Salissou *et al.* (2014) observed low prevalence of *pfcrt* resistance alleles among patients with uncomplicated falciparum malaria. In Cameroon Moyeh *et al.* (2018) observed a significant reduction of Pfcrt 76T and Pfmdr1 86Y genotypes i.e. 97.0% to 66.9% and 83.6% to 45.2% between 2003 and 2013 respectively. A review of published articles by Ocan *et al.* (2019) demonstrated the prevalence of Pfcrt 76T as follows; East Africa 48.9%, Southern Africa 18.6%, West Africa 58.3% and the prevalence of Pfmdr1 86Y in those parts of Africa were; 32.4%, 36.1%, and 52.2% respectively.

Likewise, recent studies in many other African countries reported marked reduction in the prevalence of chloroquine resistant strains of *P. falciparum* indicating gradual return of the genotypes sensitive to chloroquine and other 4-aminoquinoline derivatives (Xu C *et al.* 2018, Yao *et al.* 2018, Thomsen *et al.* 2013, Duah *et al.* 2013, Gbotosho *et al.* 2012). It is to be mentioned that, chloroquine sensitive strains is becoming the predominant strains of *P. falciparum* in locations where chloroquine has been almost completely removed from the market, including the private sector for 6–10 years (Alam *et al.* (2017).

Prevalence of *pfmdr1* N86Y mutations in Bangladesh in this study (20.69%) is in moderate range which is in congruence with reports published in past few years. (Akter *et al.* 2012, Alam *et al.* 2017). This rate for 5 Sub-Saharan African countries in this study is even lower (2.44%) which is also lower than the finding of few reports published in the recent past (Moyeh *et al.* 2018, Ocan *et al.* 2019).

Artemisinin is the keystone of present day fight against malaria. Since introduction of artemisinin combination therapies (ACTs) as 1st line treatment of uncomplicated falciparum malaria by majority of the endemic countries, a giant leap in malaria control was observed. Global malaria deaths dropped 62% between 2000 and 2015 and a cumulative total of 6.8 million lives were saved (WHO 2017[b]). But,this achievement is threatened by development of *P. falciparum* resistance to artemisinin which was first reported in 2008 in the Thai-Cambodia border of Greater Mekong Sub-region followed by reports of evidence of failure of a number of ACTs in that region.

In 2014 Ariey *et al.* identified a molecular marker for artemisinin resistance (*PfKelch13*) that is helping global surveillance of artemisinin resistance. By 2018, around 200 non-synonymous mutations in the K13 gene have been reported including 9 validated and 11 candidate/associated with artemisinin resistance (most of them are in GMS) identified through correlation with delayed parasite clearance in clinical studies and/or *in vitro* drug sensitivity assays (WHO 2018[d]). Although multiple K13 mutations have arisen independently, successful multidrug-resistant parasite genotypes are taking over and threaten to spread to India and Africa. (Woodrow *et al.* 2017). A molecular epidemiological study suggested that the dominant artemisinin-resistant *P falciparum* C580Y lineage probably arose in western Cambodia and then spread to Thailand and Laos, outcompeting other parasites and acquiring piperaquine resistance. The emergence and spread of fit artemisinin-resistant *P falciparum* parasite lineages, which then acquire partner drug resistance across the Greater Mekong subregion, threatens regional malaria control and elimination goals (Imwong *et al.* 2017). Few non-synonymous K13 mutations have also been reported in Africa.

Studies in Myanmar, the eastern neighbor of Bangladesh report high prevalence of K13 F446I mutation associated with an intermediate rate of delayed parasite clearance (WHO 2017[c]). Few non-synonymous mutations in *pfk13* gene have been reported in Indian provinces of Arunachal, Tripura, Mizoram and West Bengal having borders with Bangladesh (Mishra *et al.* 2015). Although, very few molecular surveillance studies have been conducted in Bangladesh, only one non-synonymous mutation is reported so far.

In the present study, we collected blood samples from two groups of Bangladesh Armed Forces members, 1st group working in Chittagong Hill Tracts and Ramu, an endemic area bordering with both Myanmar and India while the 2nd group working in Sub-Saharan African countries. These people also travel between those two areas. We sought to investigate whether these people are picking up artemisinin resistant strains of *P. falciparum* from either of endemic areas.

We employed a nested PCR protocol (as described by Menard *et al.*2013) to amplify *Pfk13* gene followed by sequencing. Sequencing of *pfk13* gene was done in 69 isolates, 29 from CHT Bangladesh and 40 from Africa.

No SNP (single nucleotide polymorphism) was observed among the isolates from Bangladesh. This result is in consistence with the findings of recent studies searching *pfk13* mutations in Bangladeshi samples. Alom *et al.* (2017) also observed no mutation in *pfk13* gene among 10 isolates from Bandarbans district of Bangladesh which were related with prolonged parasite clearance. WHO did not report *pfk13* mutation in Bangladesh till 2018 (World Health Organization 2018[c]). However, there was a report of *pfk13* A578S mutation in two samples from Khagrachari district of Bangladesh in 2014 (Mohon *et al.* 2014).

K13 propeller mutations were observed in 9 (22.50%) out of 40 African samples (blood collected from Bangladeshi troops working in Africa) underwent sequencing in this study. Madamet *et al.* (2015) found a similar proportion of K13 mutation (23.8%) in *Pf* positive isolates from the Union of Comoros. However, Proportion of k13 mutant isolates among *P. falciparum* positive samples undergoing sequencing shows wide range of variation (Mvumbi *et al.* 2017, Zaw *et al.* 2018, Ocan *et al.* 2019[b]).

In the present study, we report 5 non-synonymous and 3 synonymous mutations in *pfk13* gene for the first time. Non-synonymous mutations are A617P, Y616F, S491F, N458k and Y616F and synonymous mutations are F614F, I616I and K503K. All of these new non-synonymous and synonymous mutations have been detected in the samples collected from Bangladeshi troops working in D R Congo. The S491F mutation was detected in two of the isolates. Mutations F614F and A617P was detected in a single isolate from Congo.

The new mutations in *pfk13* gene we have reported in this study were never reported in Congo or any other country in Africa or elsewhere. However, recent studies in Congo reported few other non-synonymous *pfk13* mutations i.e. F495L, S522C, V520A, M476K, N523T and E509D (Mvumbi *et al.* 2017), A578S (Mayengue *et al.* 2018).

In this study we also found the common African mutation A578S in a sample collected from a Bangladeshi soldier working in West African country of Ivory Coast. A578S is the most frequent allele observed in Africa, although it has not been associated with clinical or *in vitro* resistance to artemisinin (WHO 2018[d]). In KARMA (Artemisinin Resistance Multicenter Assessment) study Menard *et al.* (2016) observed this mutation in 41 samples from Africa.

In this study we did not get any mutation in samples from Liberia, Mali and Central African Republic though studies reported presence of few non-synonymous mutations in those countries (WHO 2018[d]). As a matter of fact, we could collect very small number of samples from those 3 countries. As per the latest update of WHO, in Africa, non-synonymous mutations are still rare and highly diverse. However, a number of mutations, including some associated with delayed clearance in the GMS (in particular C580Y), have been reported in Africa (WHO 2018[d]). Menard *et al.* (2016) also identified 150 distinct alleles, 92% of which were found in only one or two samples. Apart from A578S, V589I, S522C, V534A, F583L, and G665C, most alleles were Africa-specific and localized.

In this study we did not find any of the *pfk13* mutations, validated or candidates/ associated with artemisinin resistance identified through correlation with delayed parasite clearance in clinical studies and/or *in vitro* drug sensitivity assays as recognized by WHO so far (WHO 2018[d]).

We tried to find out whether there was any statistical relation existed between presence of *Pfk13* mutation and duration of fever the patients suffered. However, Chi-square test revealed no statistical relation between those two variables ($p > 0.05$). In this study we could not attempt to find out the influence of different mutant alleles on the parasite clearance time because of logistic difficulties in field condition in conflict ravaged areas of Africa. We also sought to find out whether there was any statistical relation between coexistence of mutation in *pfk13* gene and *pfmdr1* or *pfcrt* gene among the isolates, but Chi-square test revealed no existence of such relation.

In the present study we collected and analyzed data on some variables related to demography of the respondent soldiers. Few important demographic features of the study population are, about 84% of the respondents were from Bangladesh Army, more than 50% of them were from the age group 21-30 years and 72.62% of them were engaged in job like general military duty. The main reason behind these kinds of demographic dispositions is that comparatively younger soldiers of Bangladesh Army are usually sent to CHT and UN missions primarily on general military duty.

In this study all 252 malaria patients were followed up for few clinical outcome variables like duration of fever, number of fever episodes, failure of drug, referral, length of hospital stay etc. Statistical test reveals that all of these outcome variables were associated with the 1st line antimalarial drug used ($p < 0.05$). Not surprisingly ACT shows marked advantage over quinine in terms of efficacy as assumed through those variables. Our results are in congruence with the findings of studies conducted by Achan *et al.* (2009) and Yeka *et al.* (2013).

We had two groups of respondents basing on mefloquine prophylaxis In this study, one group comprising Bangladeshi troops working in endemic African countries under 750 mg mefloquine per person per week as a prophylactic dose against malaria and the other group comprising troops working in endemic CHT Bangladesh not under any kind of prophylactic dose. Both the groups were followed on outcome variables like duration of fever, number of fever episodes, drug failure and mutation in *pfmdr1.* In statistical analysis none of these variables except ‘number of fever episodes’ was fond to be associated with prophylactic mefloquine. As a matter of fact studies on efficacy and tolerability of mefloquine as a prophylactic drug report varying range of results. Croft *et al.* (2000) reported a trial comparing mefloquine with placebo showed mefloquine prevented malaria episodes in an area of drug resistance. Gonzalez *et al.* (2018) reported that mefloquine resulted in little or no difference in the incidence of clinical malaria episodes during pregnancy. However WHO suggests that, no antimalarial prophylactic regimen gives complete protection, but good chemoprophylaxis (adherence to the recommended drug regimen) significantly reduces the risk of fatal disease (WHO 2017).

Within purview of this research work, data was analyzed of a Bangladeshi military contingent of 738 men deployed in Liberia and monitored for 1 year (June 2012 - May 2013) for 'incidence of malaria under mefloquine prophylaxis. The incidence rate of malaria was 133 (180 per thousand in 1 year). According to World Malaria Report 2014 (WHO 2014[c]), malaria incidence rate in Liberia in 2013 was 290 per thousand population. Contributing factors for a pretty low malaria incidence rate in Bangladeshi troops in Liberia, may be high compliance of the troops to preventive practices and measures and the routine consumption of prophylactic dose of mefloquine. All the cases diagnosed by microscopy/RDT were found to have *P. falciparum* infection. This is consistent with World Malaria Report 2014, country profile of Liberia (WHO 2014[c]), where it is stated that 100% malaria cases in Liberia had *P. falciparum* infection. Here we also tried to find out relationship between different variables used in data collection. Length of febrile period was found to be associated with parasite count ($P=0.04$). No association was detected between febrile period and time spent before starting antimalarial drug and also between range of temperature and parasite count ($p>0.05$).

In the present study we analyzed malaria related Knowledge Attitude and Practice (KAP) data collected from all 252 respondent soldiers suffered from malaria and provided blood samples.

Almost 92% of the respondent soldiers identified mosquito bite as the route of transmission of malaria to human body while 31% of them think that other insects may also contribute in the process of transmission. Many community based KAP studies in Asia and Africa found similar state of knowledge among population (Enato *et al.* 2007, Adedotun *et al.* 2010, Hossain *et al.* 2010, Bashar *et al.* 2012, Bhattacharyya 2014, Astatkie 2010 and Bhattacharyya 2015).

While answering a question about the efficacy of prophylactic mefloquine (MQ) 75.8% of the 'respondents under MQ prophylaxis (working in Africa)'were affirmative about the efficacy of the drug whereas, among the respondents who were not under mefloquine prophylaxis (working in CHT Bangladesh) this proportion was only 39.4%. The difference is statistically significant ($P=0.00$). However, 64.7% of all

respondent troops were affirmative about the efficacy of MQ prophylaxis. In a KAP survey among Sri Lankan security forces personnel returning from peacekeeping missions in malaria endemic countries, Fernando *et al.* (2016) found that 98.9 % of the respondents were well aware of the need for chemoprophylaxis during their overseas stay.

Over the opinion of the cause of drug resistance in malaria, the respondents were divided in number of groups. About 32% of them opined that malaria parasite develops resistance naturally while others blame low quality medicines (23.5%), irregular intake of drugs (12.35%), environmental change (9.16%) etc. for drug resistance. While choosing the most important preventive measure against malaria, 62.30% of the respondents chose the use of mosquito net followed by protective clothing (9.92%), indoor spray (6.35%), outdoor Spray (5.95%) and so on. In a KAP study in Bangladesh Bashar *et al.* (2012) found that, the majority of 468 respondents believed that bed nets were the main protective measure against malaria. While reviewing the prevention and control strategies for malaria, Tizifa *et al.* (2018) noted that use of long-lasting insecticide-treated nets and indoor residual spraying had resulted a decline in the incidence and prevalence of malaria in Sub-Saharan Africa.

We sought to find out the respondents' source of information and tried to correlate the sources with correctness of their understanding about malaria. The number one source of malaria related information of the respondents was health personnel (55.16%), followed by superior authority (28.97%), self-study (4.37%) and so on. Statistical analysis shows that, the correctness of their understanding about malaria transmission, cause of resistance and preventive priorities was associated with their source of information ($p < 0.05$). In a study conducted to assess Korean soldier' knowledge on malaria and malaria preventive behavior, Hong *et al.* (2017) showed that level of education, educational experience on malaria and ranks like corporal, and sergeant were observed to be associated with the level of knowledge of malaria. Moreover, knowledge of malaria and malaria education experience were the factors that influenced malaria prevention behavior and practice.

In this study few antimalarial preventive activities practiced by the respondent soldiers were recorded and analyzed. Mosquito net was used by 90%-100% Bangladeshi troops in study areas. Day and night usage of bed net was between 60% – 90% in most of the countries they worked. This rate of bed net usage is pretty high as compared to the usage in common endemic areas in Asia and Africa where it ranges from 13% to 78% (Musoke *et al.* 2015, Bhattacharyya H. 2015, Khairya *et al.* 2017, Anene-okeke *et al.*2018, Khanam *et al.* 2018). But, the proportion of insecticide treated bed net (ITN and LLIN) usage among Bangladeshi troops in both Bangladesh (40%) and Africa (5%) is much lower than that of prevailing bed net usage (78%, Khanam *et al.* 2018) in endemic districts in Bangladesh. One important reason remains behind this low usage of insecticide treated bed net is that troops deployed in remote areas had limited or no access to ITN/LLIN. ITN and LLIN usage also vary from country to country (Adedotun *et al.* 2010, Anene-okeke *et al.*2018, Tizifa *et al.* 2018, Khanam *et al.* 2018).

Protective clothing like full sleeved shirts, pants and shocks in the evening and early morning were used every day by majority (70%-95%) of the troops working in different countries except in CAR. As a matter of fact, use of such clothing is a part of their military discipline. Soldiers working in CAR are found to be little reluctant in regular use of protective clothing probably due to high environmental temperature and humidity. However proportion of frequent use of protective clothing by them is almost 90%. In civilian population use personal protective measures is scarce or as low as 15% (Borkar *et al.* 2017).

A wide range of variation is observed (5%-100%) in using mosquito repellent topical lotion/oil and indoor spray/coil by the troops working in different countries. Outdoor mosquito-cidal spray was arranged by the military authorities in the camps, regular/frequent practice of which was reported by the troops at quite high proportions (80% - 100%). In civilian population in endemic areas of Asia and Africa use of outdoor spray varies between a wide range (Soan and Chand 2006, Adedotun *et al.* 2010, Astatkie 2010, Khairya, *et al.* 2017).

We tried to find out the relation of preventive behavior of the troops with their source of information. In statistical analysis, use of protective clothing, insecticide treated bed net and use of mosquito repellent by the troops were found to be associated with their source of information ($p < 0.05$).

This study, first of its kind to be conducted in members of Bangladesh Armed Forces deployed in malaria endemic areas of both Bangladesh and Africa, attempted to investigate and compare molecular and epidemiological pattern of antimalarial drug resistance. It revealed 5 new non-synonymous and 3 synonymous mutations in *pfk13* gene found in its African samples. Further study is required to see the relationship of these new mutations with delayed parasite clearance and eventually artemisinin resistance. Further study is also required to examine the potential transportability of drug resistant strains of *pf* malaria between endemic areas.

Conclusion

The *P. falciparum* DNA was confirmed in 35 (37.23%) Bangladeshi and 45 (28.48%) African samples. The '*pfcrt* (K76T) mutation' that confers resistance to chloroquine, was detected in 93.10% Bangladeshi and 29.27% African samples. The '*pfmdr1* (N86Y) mutation' that confers resistance to lumifantrine and mefloquine, was detected in 20.69% Bangladeshi and only 2.44% African samples. None of the Bangladeshi samples had mutation in k13 propeller domain. On the other hand, 9 (22.50%) African samples exhibited *pfk13* mutations including 5 non-synonymous and 3 synonymous mutations, reported for the first time. All of these new non-synonymous mutations namely A617P, Y616F, S491F, N458k and Y616F and synonymous mutations namely F614F, I616I and K503K were found in samples from D R Congo. Mutations F614F and A617P were detected in a single isolate and S491F was detected in two of the isolates. The most common African mutation A578S was detected in a sample from Ivory Coast. None of the *pfk13* mutations, so far recognized to be associated/candidate or validated as artemisinin resistance marker by World Health Organization (WHO), was detected in this study. While analyzing clinical data

it was found that, duration of fever, number of fever episodes, failure of drug, referral and length of hospital stay were associated with the 1st line antimalarial drug used ($p < 0.05$). Epidemiological data in this study revealed a yearly incidence of 180 cases of malaria per thousand population under mefloquine prophylaxis in Liberia, although variables like duration of fever, drug failure and mutation in *pfmdr1* were not associated with this prophylaxis in all the study areas. While analyzing data on knowledge attitude and practice (KAP) it is found that troops under mefloquine prophylaxis were more confident on the efficacy of this prophylactic drug ($P=0.00$). Majority (92%) of the troops could identify mosquito bite as the route of malaria transmission while 62.30% chose bed net as the most important preventive measure against malaria. The correctness of their understanding about malaria transmission, cause of resistance and preventive priorities, was associated with their source of information ($p < 0.05$). More than 90% of the troops used bed net as a protective measure although only 19% of them used insecticide treated net. Regular use of protective clothing to prevent mosquito bite by the soldiers in different study areas varied between 70% and 95%. A routine practice of outdoor mosquito-cidal spray was reported by more than 80% troops working in different countries. Preventive behavior of the troops like use of protective clothing, insecticide treated bed net and mosquito repellent were found to be associated with their source of information ($p < 0.05$).

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Appendix I

Note: This questionnaire was translated into Bangla for collection of data.

Questionnaire

Title: Antimalarial drug resistance and associated genetic polymorphism of *Plasmodium falciparum* in members of Armed Forces of Bangladesh working in endemic areas at home and abroad

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Code:

Please give tick mark/fill up where necessary:

Verbal/Written consent taken: O Yes O No

Date: ☐ ☐ ☐

Patient's Personal identity

No:…………….Rank…………….Name:……..……………….. unit:……………

Basic information

1. . Present Country of duty:……………………………………

2. Location/Camp: …………………………………. 3.District/Area:…………

4. Age :………..Yrs. 5. Sex:……… 6. Religion:………

Questions

7. Nature of Job: O General duty O Office Job O Cleaning Job
O Driving O Other : Please Specify……

8. How long are you in this mission/CHT? …………………months.

9. How Malaria is confirmed this time? O ICT/RUT O Blood Film (Slide)

10. How many days did fever continue?………………………days.

11. Daily rise of temperature:……………………………. times.

12. Total rise of temperature in this episode:……………………. times.

13. Range of temperature :…………^{O}F to ………………. ^{O}F.

14. Whether the patient was referred or not : O Not refd

O Refd to Level I O Refd to level-II O Refd to level-III

15. Duration of Hospital stay:………………days.

16. Rest given for:………………………… days after discharge.

17. Antimalarial drug started this time:

O Tab. Chloroquine O Tab/Injection. Quiniue (Jesoquine)
O Tab. Fansider O Tab. Artemether+lumifentrine
O Injection. Artemether O Other: Please specify:………………

18. Failure of 1st antimalarial drug this time: O Yes O No

19. Second antimalarial given in case of failure of the 1st one:

O	Tab. Artemether+lumifentrine	O	Injection. Artemether
O	Tab/Injection. Quinine	O	Other : Please specify………
O	1st one not failed		

20. How many times did you suffer from malaria in this mission/CHT tenure?
………… .. times.

21. History of failure of antimalarial in this mission/CHT tenure**:**
O Yes O No

22. That/those failure happened to following antimalarial :

O Chloroquine O Quinine O Fansider
O Artemether+lumifentrine O Other : Please specify…

23. That/those episode of Malaria was/were Cured by:

O	Tab. Artemether+lumifentrine	O	Injection. Artemether
O	Tab/Injection. Quinine	O	Other : Please specify…

Preventive practice, Patient's View:

24. Is there any profylaxis dose given, to prevent malaria in this mission/CHT tenure?

O Nil O Yes, Mafloquice O Other, Please Specify………………

25. How frequently is this dose given?

O Daily O Weekly O Monthly

26. When do you use bed net?

O Day and night O Only night O Nil

27. Do you use insecticide treated bed net? O Yes O No

28. Do you use mosquito repellents like coil, aerosol?

O Every day O Frequently O Rarely O Nil

29. Do you use repellent oil/lotion like odomos on skin?

O Regularly O Frequently O Rarely O Nil

30. Do you wear protective clothing like full sleeves, stockings etc to prevent mosquito bite?

O Regularly O Frequently O Rarely O Nil

31. Use of fogger/cimbush spray in your camp/area**:**

O Everyday O Frequently O Rarely O Nil

Malaria awareness, Patient's View:

32. How malaria is transmitted from one person to another?

O Only mosquito bite O Mosquito and other insect bite
O Nasty environment O By Cough/sneeze
O Any other: Please specify………………………………….

33. What action should get the 1st priority to prevent Malaria?

O Use of Mosquito net O Fogger spray
O Use of coil/aerosol O Use of clothing like full sleeves
O Use of lotion/cream like odomos O Any other : Please specify……

34. Dose weakness/malnutrition has got relation with getting Malaria?

O Yes O No

35. Can high protein diet prevent Malaria?

O Yes O No

36. How/where should malaria patient be treated?

O At home/room O At hospital O homeopathy
O kobiraji

37. Can Mafloquine prevent malaria? O Yes O No O Sometimes

38. Is there any drug resistant malaria in this area? O Yes O No

39. If yes, then resistant to what drug?

O Chloroquine O Quinine O Artemether
O Fansider O Other : Please specify……………

40. Why does this resistance occur?

O Capacity of the organism O Low quality of medicine
O Irregular intake of medicine O Change of environment
O Other: Please specify…………………...

41. How do you get knowledge about malaria?

O Own study O Motivation by doctor/health worker
O Motivation by higher commander O Other: Please specify……