Epidemiology of malaria in Bangladesh: detection and identification of genetic and molecular variation in *Plasmodium falciparum*

Thesis submitted in fulfillment of the requirement for the degree of
DOCTOR OF PHILOSOPHY

By
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TO

MY BELOVED PARENTS

AND

RESPECTED SUPERVISORS

Declaration

It is my greatest honor and privilege to declare that the dissertation on **"Epidemiology of malaria in Bangladesh: detection and identification of genetic and molecular variation in *Plasmodium falciparum*"** is carried out by me under supervision and guidance of Professor Dr. Hamida Khanum, Parasitology Branch, Department of Zoology, University of Dhaka and Dr. Rashidul Haque, Senior Scientist, CVS and Head, Parasitology Laboratory, ICDDR,B, Mohakhali, Dhaka for fulfillment of the degree of Doctor of Philosophy under the University of Dhaka, Dhaka-1000, Bangladesh.

I left no stone unturned to make the dissertation unique, informative and comprehensive one with the sincere co-operation and valuable guidance of my supervisors. In this regard, I would like to confirm that the research works documented and analyzed in this dissertation are original and had never been submitted for any other degree.

Sincerely,

Shafiul Alam

.....

Mohammad Shafiul Alam

CERTIFICATE

We certify that the thesis entitled **"Epidemiology of malaria in Bangladesh: detection and identification of genetic and molecular variation in *Plasmodium falciparum*"** submitted by Mohammad Shafiul Alam, for the degree of Doctor of Philosophy in Zoology of the University of Dhaka, Bangladesh, embodies the record of original investigations carried out by him under our supervision.

7.1.15

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Contents

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…….

ABSTRACT

In the present study, different aspects of malaria in Bangladesh were studied including its epidemiological aspects, knowledge, attitude and practices of the people; development of two high level molecular diagnostic methods for the most virulent malaria parasite (*Plasmodium falciparum*) followed by its genetic diversity.

In the epidemiological study during 2010-13, a total of 1,64,055 malaria cases were reported from 13 endemic districts of Bangladesh. The overall mean female: male ratio of annual malaria incidence was1:1.42. Malaria caused by *P. falciparum* was found dominant in Bangladesh which was 94.2% of total malaria cases. Only 5.5% cases were reported to be caused by *P. vivax*. Remaining infection was caused by *P. falciparum* and *P. vivax* mixed infection. A total of 99 patients died during this period (2010-13). Quiet opposite to the malaria incidence, death of female (51) was slightly more than death of male (48). Highest number of death was 37 in 2010 followed by 36 in 2011.

Bandarban contributed on average 31.3% cases reported 30.9%, 31.1%, 28.7% and 35.2% in respective years from 2010-13. Bandarban also reported the highest *P. falciparum* incidence during this period. On the other hand Rangamati recorded highest *P. vivax* infected malaria cases in 2010 and 2011 but Cox's Bazar in 2012 and 2013. Malaria incidence was found highest during June- July of a particular year. In Bangladesh, during 2010-13 the average annual cases incidence was 41,014.

The KAP study was conducted in six different districts of Bangladesh, three districts each from both malaria endemic and non-endemic areas. In the endemic region more than 50% of the individuals had malaria within past five years of the study. Malaria transmission was known to less than 10% of the respondents. The correct symptom of malaria was known to 27% of the respondents in the non-endemic region whereas nearly 70% knew that onset of fever with shivering as the primary symptom of malaria. However, knowledge on malaria transmission and symptom has been observed to be improved according the educational status of the respondents. However, only about 14% of the distributed ITNs had been reported to be re-treated

with insecticide in the endemic region. Mostly, mosquito coil was observed to be common among the respondents. Use of insecticide

For establishment of molecular methods, blood samples were collected from Khagrachari district of Chittagong Hill Tracts. After having the successful amplification by the newly developed real-time PCR method detection limit was established following the typical amplification curve. The new real-time PCR could detect less than 1 parasite/uL from clinical sample. The melt peak for *P. falciparum* was found at 74.5 °C from the corresponding positive controls and 50.3% samples were found positive. For the detection of *P. falciparum,* newly developed real-time PCR assay had 97.1% (95% CI: 93.3-99) sensitivity and 97.6% (95% CI: 94-99.3) specificity respectively. According to parasite count (per uL) by microscopy, newly established real-time PCR provided excellent result as100% sensitivity was found in 6 out of 7 groups.

For molecular diagnosis of malaria by nucleotide amplification, a new primer set was designed targeting the18S rRNA gene for the detection of *P. falciparum* in blood samples collected from the field. The efficacy of LAMP using the new primer set was assessed in this study in comparison to that of a previously described set of LAMP primers as well as with microscopy and real-time PCR as reference methods for detecting *P. falciparum*. The new LAMP assay was found to be 99.1% sensitive compared to microscopy and 98.1% when compared to real-time PCR. Meanwhile, its specificity was 99%and 100% in contrast to microscopy and real-time PCR. The new LAMP method can detect at least 5 parasites/µL of infected blood within 35 min. Pre-addition of hydroxy napthol blue (HNB) in the LAMP reaction caused a distinct color change, thereby improving the visual detection system. Thus, the new method is sensitive and specific, can be carried out in a very short time, and can be used in the field level for molecular diagnosis of *P. falciparum*.

This study provides valuable information on genetic diversity of in *P. falciparum* Bangladesh. PCR amplification of template DNA and analysis of region II of *GLURP*, central polymorphic region of *MSP2* (3D7 and FC27 allelic families), and block 2 of *MSP1* (K1, MAD20 and RO33 allelic families) was performed in this study.

The present study found that the frequency of MSP1 allelic families was higher than MSP2 families in Bangladesh, although the number of alleles remained higher in MSP2. Almost equal dominance was shown by K1 and MAD20 for MSP1. A total of 168 clones were found by MSP1 specific PCR of which 93 (55.4%) were from Chittagong Hill Tracts (CHT) areas and remaining 75 were of (44.6%) from non-CHT areas. In MSP2 specific PCR 128 clones were found of which 66 (51.6%) were of non-CHT areas and remaining 62 (48.4%) were of CHT areas. A total of 141 clones belonging to 13 allelic families were found by GLURP specific PCR of which 72(51.1%) were of CHT areas and remaining 69 (48.9%) were of from non-CHT areas.

Being monomorphic clones of RO33 allelic family of MSP1 was found highest (31.5%) in overall distribution. However, the contribution of non-CHT and CHT areas on RO33 frequency distribution were 12.3% and 19.27%. In Fc27 allelic family the highest contribution (13.1%) in frequency distribution was reported in 276-300 bp category from overall data. The highest contribution of Fc27 frequency distribution reported from non-CHT was also in 276-300 bp groups (8.5%) and from CHT in 301-350 bp group (8.5%). GLURP allelic families were ranged in between 400-1050 base pairs with highest frequency (26.9%) in 551-600 bp group in overall data, in non-CHT areas 16.2% and 10.8% in CHT along with 901-950 bp group, respectively.

Mean multiplicity of infection (MOI) of MSP1, MSP2 and GLURP was 1.51, 1.25 and 1.08, respectively in overall samples. These were found 1.44, 1.29 and 1.11 respectively in non-CHT areas and 1.58, 1.22 and 1.04 respectively in CHT areas. The expected heterozygosity (H_E) of MSP1, MSP2 and GLURP in non-CHT areas was found 0.84, 0.90 and 0.78 respectively. These values for CHT were 0.76, 0.93 and 0.86 respectively and in over all data 0.80, 0.93 and 0.83 respectively. Although, it is believed that in areas where malaria endemcity is declining the heterozygosity of *P. falciparum* genotypes will also decrease. However, this is found different in Bangladesh. This is possible that there may be a good number of asymptomatic malaria cases across the country or there may be a large number of unreported malaria cases exist in the endemic population which may eventually contribute in the high genetic diversity as observed in the present study.

ABBREVIATIONS

ACT	Artemesinin Based Combination Therapy
AIDS	Acquired immune deficiency syndrome
API	Annual Parasitic Incidence
ASO	Allele-specific oligonucleotide
BDT	Bangladeshi Taka
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BRAC	Bangladesh Rural Advancement Committee
B*st*	*Bacillus stearothermophilus*
CDC	Centres for Disease Control
CHT	Chittagong Hill Tracts
CI	Confidence interval
CT	Cycle threshold
DDT	Dichloro di phenyl tri chloro ethane
DGHS	Directorate general of Health Services
DOI	Digital object identifier
DNA	Deoxy ribonucleic acid
DNTP	Deoxy nucleotide triphosphates
EDTA	Ethylenediaminetetraacetic acid
EIR	Entomologic inoculation rates

ELISA	Enzyme linked immunosorbent assay
et al.	*et alia* (and others)
GFATM	Global Funds to Fight AIDS, Tuberculosis and Malaria
GLURP	Glutamate rich protein
GOB	Government of Bangladesh
HABP	High activity binding peptide
HNB	Hydroxy naphthol blue
HRP	Histidine-rich Protein
ICDDR,B	International Centre for Diarrhoeal Disease Research, Bangladesh
ICT	Immunochromatographic test
IRC	Indoor Residual Spraying
ITN	Insecticide-Treated Nets
KAP	Knowledge, Attitude and Practices
LAMP	Loop-mediated Isothermal Amplification
LLIN	Long-Lasting Insecticidal Nets
M&PDC	Malaria and Parasitic Disease Control Unit
MCWA	Malaria Control in War Areas
MEP	Malaria Eradication Programme
MOI	Multiplicity of Infection
MSP1	Merozoite surface protein 1
MSP2	Merozoite surface protein 2

NCBI	National Center for Biotechnology Information
NGO	Non-Governmental Organization
NMCP	National Malaria Control Program
NNE	North/north-east
NPP	Negative Predictive Value
NPV	Negative Predictive Value
PCR	Polymeric Chain Reaction
Pf	*Plasmodium falciparum*
PHC	Primary Health Care
PPV	Positive Predictive Value
Pv	*Plasmodium vivax*
RAPD	Random Amplified polymorphic Detection
RDT	Rapid Diagnostic Test
RFLP	Restriction Fragment Length Polymorphism
RFU	R elative fluorescence units
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SNP	Single Nucleotide Polymorphism
SPSS	Statistical Package for the Social Sciences
SSE	South/south-east
SSU	Small subunit

STATA	Syllabic abbreviation of the words statistics and data
SYBR	Trade name used in an asymmetrical cyanine dye used as a nucleic acid stain in molecular biology
Taq	*Thermus aquaticus*
TBS	Positive thick blood smears
TCI	Temperature condition index
Tm	Melting temperature
UHC	Upazila health complex
VCI	Vegetation condition index
VH	Vegetation Health
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

INTRODUCTION

Approximately 3.4 billion people were at risk of malaria globally in 2012. Five different parasite species of the genus *Plasmodium* cause malaria in humans (*Plasmodium falciparum, P. vivax, P. ovale, P. malariae* and *P. knowlesi*). *P. falciparum* is the most deadly and predominant than others. *P. falciparum* is the most dangerous compare to any other species. About 90% of estimated cases globally are due to *P. falciparum*, where majority of the burden occurs in Africa and South-East Asia (WHO 2013).

Global Situation of Malaria

According to World Malaria Report 2013 by World Health Organization, there are 97 countries and territories with ongoing malaria transmission, and 7 countries in the prevention of reintroduction phase, making a total of 104 countries and territories in which malaria is presently considered endemic. Globally, an estimated 3.4 billion people are at risk of malaria. WHO estimates that 207 million cases of malaria occurred globally in 2012 (uncertainty range 135–287 million) and 627 000 deaths (uncertainty range 473 000–789 000) (Chapter 8; Section 8.3). Most cases (80%) and deaths (90%) occurred in Africa (Figure 1.4), and most deaths (77%) were in children under 5 years of age (WHO 2013).

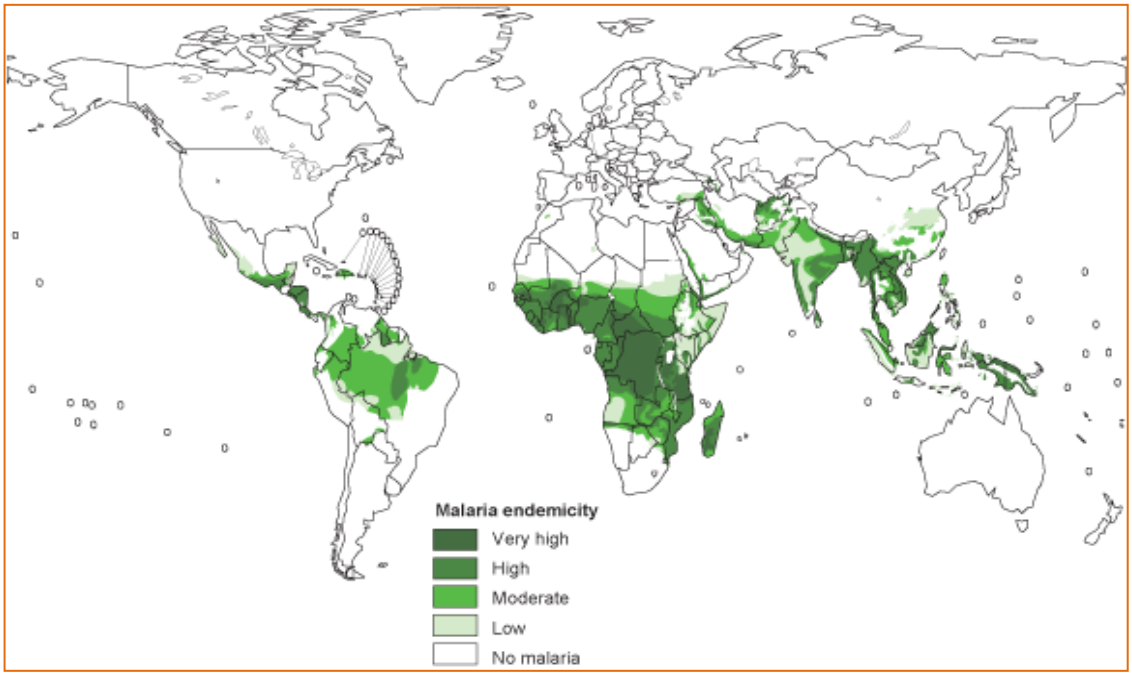


Figure 1.1: Global map of malaria distribution.

Historical Background of Malaria in Bangladesh

Malaria has infected humans for over 50,000 years, and *Plasmodium* may have been a human pathogen for the entire history of the species (Joy *et al*., 2003). Close relatives of the human malaria parasites remain common in chimpanzees (Escalante *et al*., 1998). Some new evidence suggests that the most virulent strain of human malaria may have originated in gorillas (Liu *et al*., 2010).

References to the unique periodic fevers of malaria are found throughout recorded history, beginning in 2700 BC in China (Cox *et al*., 2002). Malaria may have contributed to the decline of the Roman Empire, and was so pervasive in Rome that it was known as the "Roman fever" (Sallares 2002). The term malaria originates from Medieval Italian: *mala aria* — "bad air"; the disease was formerly called *ague* or *marsh fever* due to its association with swamps and marshland. Malaria was once common in most of Europe and North America, where it is no longer endemic, though imported cases do occur (James and Webb 2009).

Malaria was the most important health hazard encountered by U.S. troops in the South Pacific during World War II, where about 500,000 men were infected. Sixty thousand American soldiers died of malaria during the North African and South Pacific campaigns (Byrne 2008).

An early effort at malaria prevention occurred in 1896, just before the mosquito malaria link was confirmed in India by a British physician, Ronald Ross. During 1896 in Uxbridge of London, malaria outbreak prompted to the study of mosquito-malaria links, and the first efforts for malaria prevention. Massachusetts State pathologist Theobald Smith worked on collected mosquito specimens for further analysis and that citizens 1) add screens to windows and 2) drain collections of water. Carlos Finlay was also engaged in mosquito related research, and mosquito borne disease theory, in the 1880s in Cuba, basing his work on the study of Yellow Fever (Best 1993).

In the early 20th century, before antibiotics became available, Julius Wagner-Jauregg discovered that patients with syphilis could be treated by intentionally infecting them with malaria; the resulting fever would kill the syphilis spirochetes, and quinine could be administered to control the malaria. Although some patients died from malaria, this was considered preferable to the almost-certain death from syphilis (CDC 2013).

The first successful continuous malaria culture was established in 1976 by William Trager and James B. Jensen, which facilitated research into the molecular biology of the parasite and the development of new drugs.

Although the blood stage and mosquito stages of the malaria life cycle were identified in the 19th and early 20th centuries, it was not until the 1980s that the latent liver form of the parasite was observed. The discovery of this latent form of the parasite explained why people could appear to be cured of malaria but suffer relapse years after the parasite had disappeared from their bloodstreams.

Throughout history, the contraction of malaria (via natural outbreaks as well as via infliction of the disease as a biological warfare agent) has played a prominent role in the fortunes of government rulers, nation-states, military personnel, and military actions. "Malaria Site: History of Malaria During Wars" addresses the devastating impact of malaria in numerous well-known conflicts, beginning in June 323 B.C. That site's authors' noted: "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" (CDC 2013).

Significant financial investments have been made to fund procure existing and create new anti-malarial agents. During World War I and World War II, the supplies of the natural anti-malaria drugs, cinchona bark and quinine, proved to be inadequate to supply military personnel and substantial funding was funnelled into research and development of other drugs and vaccines. American military organizations conducting such research initiatives include the Navy Medical Research Center, Walter Reed Army Institute of Research, and the U.S. Army Medical Research Institute of Infectious Diseases of the US Armed Forces.

Additionally, initiatives have been founded such as Malaria Control in War Areas (MCWA), established in 1942, and its successor, the Communicable Disease Center (now known as the Centers for Disease Control) established in 1946. According to the CDC, MCWA "was established to control malaria around military training bases in the southern United States and its territories, where malaria was still problematic" and, during these activities, to train state and local health department officials in malaria control techniques and strategies (CDC 2013).

Recent trend of Malaria in Bangladesh

Malaria is one of the major public health problems in Bangladesh. Out of 64 districts, 13 bordering districts in the east and northeast facing the Indian states of Assam, Tripura and Meghalaya and part of Myanmar belong to the high-risk malaria zone. A total of 14.7 million populations are at high-risk of malaria in the country, although there is sporadic incidence of malaria in other parts of the country. The Annual Parasitic Incidence (API) was 4.2 in 2002 (http// www.searo.who.int).

More than 98% of the total malaria cases in the country are reported from these 13 high endemic districts. The three Hill tracts Districts (Bandarban, Khagrachari and Rangamati) and Cox's Bazar district report more than 80% of the malaria cases and deaths every year. These areas experience a perennial transmission of malaria with two peaks in pre-monsoon (March-May) and post-monsoon (September-November) periods. There are also reports of outbreaks from bordering districts in north and north-east. Both falciparum and vivax malaria are prevalent in the country of which the number of falciparum cases are 75% of the total cases in recent years that may be due to increasing drug resistance.

The first line drug chloroquin has been replaced by Artemesinin based Combination Therapy (ACT) for treatment of falciparum malaria cases in 2004. An estimated 1.0 million clinical cases are treated every year while 61,495 laboratory confirmed cases were reported during 2002 from routine surveillance. During 2002, a total of 598 deaths were reported. *Plasmodium falciparum* is the predominant infection (>70%). *Anopheles dirus, An. minimus and An. philippinensis* are the principal vector. Drug resistance to chloroquine and sulphadoxine-pyramethamine was reported from areas of Chittagong Hill Tract Districts (http// www.searo.who.int).

Malaria is still a threatening disease for the people of Bangladesh. The then Government of Pakistan, initiates a Malaria Eradication Programme (MEP) in 1961. This program was successful since 1971 where the rate of death due to Malaria drops down to 4.22 per 100,000 due to the frequent use of DDT by the Malaria Eradication Programme (MEP) of the then Government of East Pakistan (Haque *et al*. 2009a). But after the Liberation war, in a country where reconstruction after war was going on, the MEP was stopped. Due to this, the death rate rose to a peak of 60.44 in 1976 (Paul, 1984).

The next year, 1977, the Malaria eradication programme (MEP) merged with PHC (Current Malaria Situation – Bangladesh, 2005) and Control program was launched and the death rate fall down to 35.87 per 100000. (Paul, 1984) In 1994 Revised malaria Control Strategy was adopted and in 1998, piloting of Roll Back Malaria started in one of the Hill Tracts Districts (http//www.nmcp.info).

Since the incidence of malaria in the eastern regions was low and there was a lack of adequate funds and programs, no control efforts maintained in the malaria endemic areas of Bangladesh. Without these control efforts, malaria cases started to increase and became epidemic in the 1990s. In the late 1990s, more than 500 deaths were reported with 70,000 laboratory-confirmed cases and 900,000 clinical cases of malaria in Bangladesh (Haque *et al*. 2009a).

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. These thirteen districts, out of the 64 administrative districts of Bangladesh, are recognized as malaria endemic. Ninety eight percent of the malaria case reports come from these thirteen districts. Three out of these thirteen districts, Bandarban, Khagrachari and Rangamati, collectively known as the Chittagong Hill Tracts (CHT) districts, report the highest incidence of malaria within the country. These thirteen districts are difficult to reach due to the hilly terrain and therefore have inadequate passive surveillance and information systems resulting in poor reporting of malaria cases by the Ministry of Health, Government of Bangladesh (Haque *et al*. 2009a).

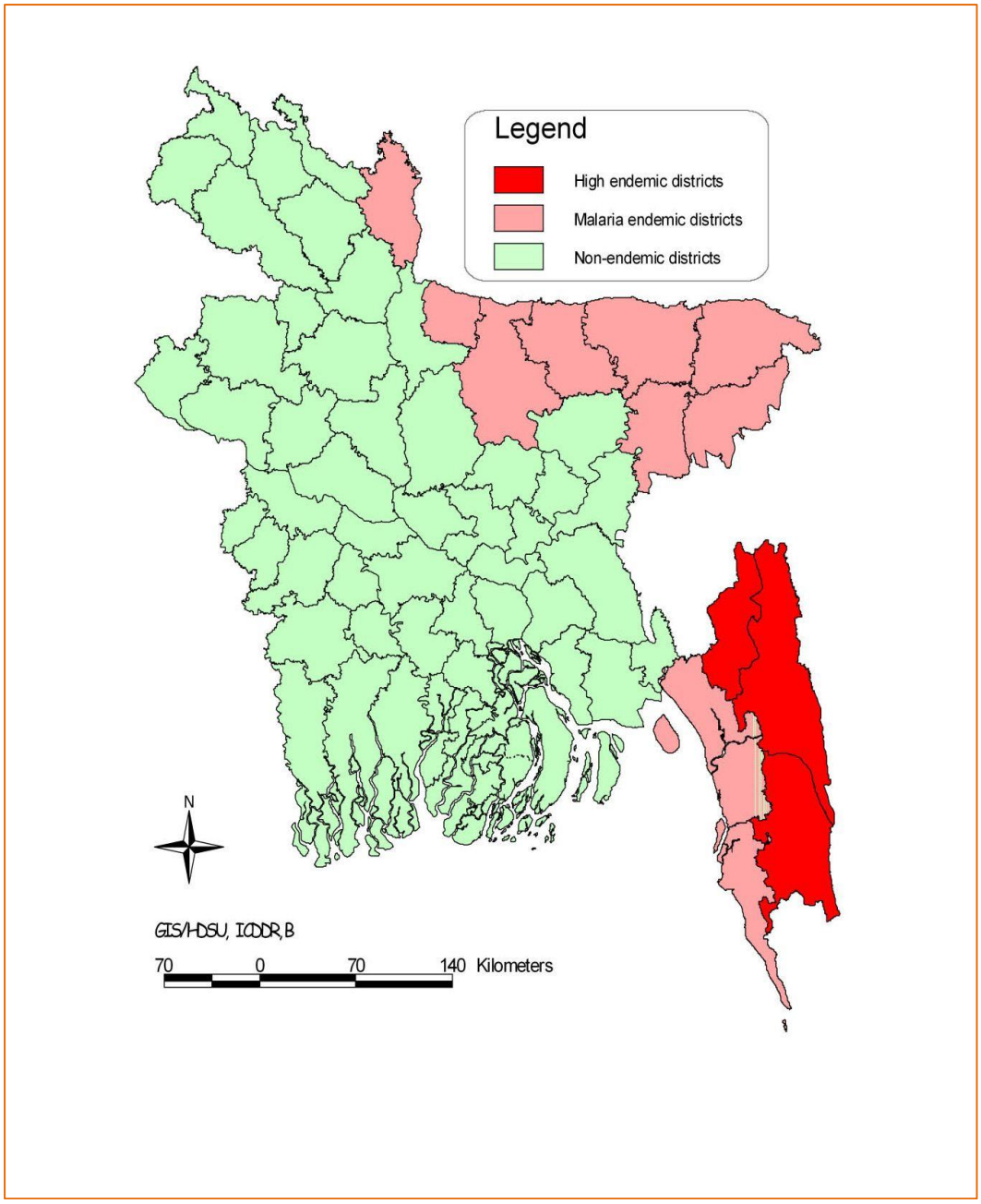


Figure 1.2: Malaria endemic areas of Bangladesh shown in a map.

Unique aspects of malaria epidemiology in Bangladesh

Population: 162,221,000

Area: 147,570 km^2/55,599 sq mi

Pop. Density: 1063/ km^2 or 2750/m^2

- Close to the epicenter of malaria drug resistance in Thai-Cambodia-Burma region
- Numerous *Anopheles* species with different biting habits

- Ability to transmit *P. vivax* and *P. falciparum* in the same human and mosquito populations and age distribution
- Seasonality and transmission patterns distinct from most of African countries
- Representative of southeast Asia
- Population density is 10-100 times more than African nations

Malaria prevalence by region

According to most recent National Malaria Prevalence Survey (Haque *et al*. 2009):

- Overall prevalence rate in Bangladesh: 3.97% (southeast 6% and northeast 0.4%)
- *P. falciparum*: 91%; *P. vivax*: 5%; mixed infection: 4%
- High prevalence in Chittagong hill tracts: 11%
- Overall prevalence in 3 hill districts: Rangamati (8%), Bandarban (12%) and Khagrachari (18%).

Knowledge, Attitude and Practices (KAP) towards Malaria

There has been considerable number of reports about Knowledge, Attitude and Practices (KAP) relating malaria and its control program all over the world. Most of these reports demonstrate the existing misconceptions both in knowledge and in practices possessed by the people at risk of malaria. Such circumstance can negatively impact malaria control strategies therefore, results might be unsatisfactory (Mazigo *et al.* 2010). It is important to understand the communities' awareness relating to causes, symptoms, mode of transmission, disease diagnosis, and proper treatment as well as their attitude the possible and decision of malaria. Moreover, disease knowledge and practice differ from community to community. For instance, different community built different types of houses. Household construction materials can significantly affect malaria incidence. Poorly constructed houses are considerably more mosquitogenic and risk for getting malaria is greater for the inhabitants (Gamage-Mendis *et al.* 1991). Furthermore, people seek treatment depending on individual perception and local counseling among themselves though fever is still not considered as a serious symptom at some parts of the world. People easily get confused over malaria symptoms and control measures (Matta *et al.* 2004). To address health equity among rural and underprivileged people such recognition is very important to develop strategies aiming to control malaria and determine the

stages of implementation of planned activities (Legess *et al.* 2007 and Adedotun *et al.* 2010).

Efficient targeting to endemic focal points is also important for successful malaria control. In Bangladesh, most of them are situated in CHT (Haque *et al.* 2011). Dense forests of this region exploited and transformed into either agricultural or shrub unused lands more than any other region of Bangladesh since 1977. Such change also alters livelihood maintenance of local people, vector population and disease incidence. Knowledge related to disease concept, transmission, treatment facilities, materials and treatment seeking behaviour have also changed over time (Ahmed *et al.* 2010). However, the potential contribution of KAP studies on malaria had not received much attention in Bangladesh. Thus, advance knowledge of the community belief and practices with respect to the disease is required to obtain and maintain its participation in surveillance and control activities.

Malaria was nearly eradicated from Bangladesh by the 1970's, but never disappeared in the eastern regions of Bangladesh associated with tea gardens and forests where *Anopheles baimaii* (previously known as *An. dirus* D) transmitted *falciparum* malaria are common. It has re-emerged as one of the major public health problems in 1990s in India as well in Bangladesh (Haque *et al.*, 2009 a; Sharma, 1996). Malaria is also a major health burden in the remote, mountainous South-eastern region of Bangladesh. Malaria transmission in Bangladesh is mostly seasonal and concentrated in the border regions with India and Myanmar. Out of 64 districts of Bangladesh, 13 are located along with the border areas with India and Myanmar (Alam *et al.* 2010).

About 98% of the total malaria morbidity and mortality reported from Bangladesh each year originated from those districts (Haque *et al.* 2009a; Ahmed *et al.*2009). In a recent study it has been found that among these 13 malaria endemic districts, the overall malaria prevalence rate was 3.1% based on Rapid Diagnostic Test (RDT). The prevalence of *P. falciparum* in the study area was 2.73% and that for *P. vivax* was 0.16%. Mixed infection of *P. falciparum* and *P. vivax* had a prevalence of 0.19%. The proportion of *P. falciparum* in all parasite positive samples was 88.6%. *P. vivax* and mixed infection had been detected in 5.2% and 6.25% of infections, respectively. In the same study overall malaria prevalence in Chittagong Hill Tracts was found11% (Haque *et al.* 2009a). However, RDT may underestimate the actual

prevalence as their sensitivity increases with the increasing parasitemia (McMorrow *et al.*, 2008). RDTs have not yet been developed that allow the user to quantify the parasite burden that might enable the clinicians to guide and assess anti-malarial therapy (Duffy and Fried 2005).

For the last 100 years, malaria has been diagnosed by microscopic examination by Giemsa-stained thick and thin blood films (Bruce-Chwatt, 1987).However, it is well documented that microscopy has limitations: it is time-consuming, and misdiagnosis of the infecting species is common if the microscopist lacks experience and/or when the parasitemia is low (Milne *et al*. 1994; Kain *et al.* 1998 and Singh *et al.* 1999). Serological diagnostic methods such as rapid diagnostic test (RDT) for *Plasmodium* specific antigen in recent time is being widely used. The advantages offered by this methods, such as the fact that a result can be obtained within half an hour by non skilled technicians, are tempered by three limitations (Moody 2002). However, RDT methods do not offer improved sensitivity over microscopy; the sensitivity decreases as parasitemia fall below 100 parasites/µl (Mills *et al.* 1999).

Recently developed Polymeric Chain Reaction (PCR) assays have various advantages over microscopy and RDT: they are highly specific and are capable of high sensitivity and as few as five parasites per microliter of blood can be detected (Moody, 2002). Real-time PCR a new methodology that employs fluorescent labels to enable the continuous monitoring of amplicon (PCR product) formation throughout the reaction—has recently been adapted to detect all four human malaria parasites indiscriminately and screen large numbers of samples (Lee *et al.* 2002; Perandin *et al.* 2004).

LAMP (loop-mediated isothermal amplification) is a novel DNA amplification method with the distinguished feature that the reaction can precede under isothermal conditions (Notomi *et al.* 2000); the LAMP reaction requires only a single enzyme, Bst DNA polymerase that can synthesize a new strand of DNA while simultaneously displacing the former complementary strand thereby enabling DNA amplifi-cation at a single temperature. Another advantage using LAMP is based on the fact that the amplification from stem-loop structures leads to the accumulation of large amounts of products of various lengths, ultimately making detection of amplified DNA much

easier. Furthermore, the by-product of the reaction, magnesium pyrophosphate, is a white-colored precipitate easily seen by the naked eye (Mori *et al.* 2001).

Genetic diversity plays an important role in the natural acquisition of immunity to malaria infections, and is also of some extent to the development of control measures. Strategies to prevent the rapid spread of parasites resistant to novel drugs or vaccines require an understanding of the population structure of the parasites. The availability of the polymorphic genetic markers such as merozoite surface proteins (*msp1* and *msp2*), combined with the relative ease of their characterization by sensitive PCR amplification from field collected samples, have made such investigations possible. The size polymorphic 1 (*msp1*) gene has been used as a molecular marker in studies of malaria transmission dynamics and host immunity in *P. falciparum* malaria. The Block 2 region of the N-terminal of the *msp1* gene is trimorphic and three allelic families: MAD20, KI and RO33 have been identified (Ferreira *et al.* 1998 and Tanabe *et al.* 1987). Similarly, alleles at the *P. falciparum msp2* locus, encoding merozoite surface protein-2 (*msp2*), fall into two highly divergent families (Smythe *et al.* 1990).

Justification of the study

1. Bangladesh is a malaria prone country. Malaria once had a significant role of mortality in the country (GoB 1977 and Paul 1984). Due to some successful interventions it is believed that the trend of malaria has been decreased in recent years. However, this needs to be documented.

2. Due to ongoing malaria control initiative the people who lives in the endemic areas have to have better knowledge on malaria transmission, treatment and prevention (Ahmed *et al.* 2009). However, currently in non endemic areas malaria related knowledge is not transmitted. As vectors of malaria (female *Anopheles* mosquitoes) are present throughout the country. A sudden malaria outbreak in non endemic areas can cause heavy morbidity and mortality. Thus, there is a need to review the knowledge about malaria transmission, treatment and prevention is required.

3. Two malaria parasite species (*Plasmodium falciparum* and *P. vivax*) are common in Bangladesh. However, *P. falciparum* is the responsible parasite to cause severe

malaria and high mortality throughout the world. In a recent time it has been shown that more than 90% of the malaria in Bangladesh is associated with *P. falciparum* infection (Haque *et al.* 2009a). Thus, *P. falciparum* was selected for the present study as this species may cause high mortality and morbidity in endemic areas.

4. In present time, real-time PCR is being used throughout the world (Perdin *et al.* 2004) in reference laboratories to detect clinical and asymptomatic malaria infection. But before initiating the present study real-time PCR for detection of malaria had been not established in Bangladesh.

5. Loop Mediated Isothermal Amplification (LAMP) is a newly developed molecular technique which has great possibility to perform in the field level as a point of care test (Poon *et al.* 2006). Few research groups around the world are now working to develop and improve this method. Thus, the study has created a great opportunity for Bangladesh to compete in the global scale in developing and improving malaria LAMP.

6. *Plasmodium facliparum* has diverse genetical variations. Genetic diversity play important role in immune-epidemiology of malaria. However in Bangladesh genetic diversity of *P. falciparum* has never been studied across the endemic areas. It is possible that epidemiological outcome may vary with different genotypes of *P. falciparum*. This investigation will also provide chances to study the allelic frequency distribution of *P. falciparum*. This might help to discriminate the parasites isolates from within a host by the PCR-based genotyping of the genetic markers.

7. It has been known that *P. falciparum* has established resistance against the antimalarial drugs (Noedl *et al.* 2009). Genotyping of *P. falciparum* by using the specific markers will provide an indication of the level of resistance to antimalarial drugs by the parasites. The development of resistance to antimalarial drugs continues to be a major problem in the treatment of *P. falciparum* infection.

Objectives:

General objectives:

1. To study the epidemiological aspects of malaria in Bangladesh;
2. Study the knowledge, attitude and practice towards malaria in Bangladesh;
3. To establish a real-time PCR method with clinical *P. falciparum* malaria samples;
4. Establishment of a new Loop Mediated Isothermal amplification (LAMP) based molecular diagnosis for *P. falciparum*; and
5. To document genetic variation of *P. falciparum* in Bangladesh.

Specific objectives:

1. Observing the variable characteristics of malaria infection dynamics in Bangladesh;
2. To observe socioeconomic factors of the malaria infected patients in the study area;
3. Study the knowledge on malaria transmission in both malaria endemic and non-endemic areas of Bangladesh;
4. Collecting information on attitude towards malaria in both malaria endemic and non-endemic areas of Bangladesh;
5. Documenting the practice towards malaria in both malaria endemic and non-endemic areas of Bangladesh;
6. To identify the *P. falciparum* by using real-time PCR;
7. To calculate the sensitivity and specificity of real-time PCR for the diagnosis of *P. falciparum*;
8. To test a new molecular detection method, Loop Mediated Isothermal Amplification (LAMP) for the identification of *P. falciparum*;
9. Improving the LAMP method for diagnosis of *P. falciparum*;
10. Enumerate the sensitivity and specificity of real-time PCR for the diagnosis of *P. falciparum*;
11. Identifying diversity of *P. falciparum* genetic markers: MSP1, MSP2 and GLURP- allelic distribution and their polymorphism in endemic areas of Bangladesh.

Hypothesis

The study has following hypotheses:

1. Malaria has a decreasing trend in recent year in Bangladesh;
2. The knowledge of the people in malaria non-endemic areas about malaria transmission and control are poor compare to endemic areas of Bangladesh;
3. Real-time PCR would have high sensitivity and specificity for the detection of clinical *P. falciparum* infection;
4. LAMP is highly sensitive and specific as like real-time PCR for detection of *P. falciparum*;
5. High polymorphism for *P. falciparum* genetic markers (MSP1, MSP2 and GLURP) exist in endemic areas of Bangladesh.

Diagnosis of Malaria

The mainstay of malaria diagnosis had been the microscopic examination of blood. Although blood is the sample most frequently used to make a diagnosis, both saliva and urine have been investigated as alternative, less invasive specimens.

Characteristics of an ideal diagnostic tool for malaria include:

- Results should be available rapidly (within one hour);
- Easy to use with minimal training requirements;
- Minimal materials with long shelf-life that do not require refrigeration or electricity;
- Reproducible results;
- Allows detection of all malaria species, including mixed infections;
- Accurate even at low parasite density (<50 parasites/microL blood);
- Allows quantification of parasitemia;
- Allows monitoring response to therapy (including detection of recrudescence or relapse);
- Revert to negative within a few days of clearing viable parasites; and
- Cost effective.

Desirable characteristics for diagnostic tests vary depending on the epidemiology of infection and goals for control in the region where the test is used. Thus far, no diagnostic tool has been developed that meets all of the above criteria.

Symptomatic Diagnosis of Malaria

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.

Blood films

The most economic, preferred and reliable diagnosis of malaria is the microscopic examination of blood films because each of the four major parasite species has distinguishing characteristics. Two sorts of blood film are traditionally used. Thin films are similar to usual blood films and allow species identification because the parasite's appearance is best preserved in this preparation. Thick films allow the microscopist to screen a larger volume of blood and are about eleven times more sensitive than the thin film, so picking up low levels of infection is easier on the thick film, but the appearance of the parasite is much more distorted and therefore distinguishing between the different species can be much more difficult. With the pros and cons of both thick and thin smears taken into consideration, it is imperative to utilize both smears while attempting to make a definitive diagnosis (Figure 1.6).

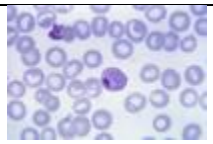
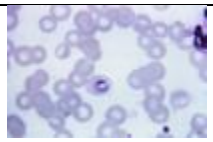
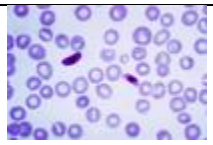
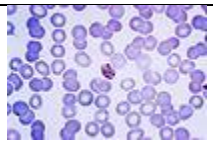
Species	Appearance	Periodicity	Liver persistent
Plasmodium vivax		tertian	Yes
Plasmodium ovale		tertian	Yes
Plasmodium falciparum		tertian	No
Plasmodium malariae		quartan	No

Figure 1.3: Comparison of blood films showing different types of *Plasmodium* spp.

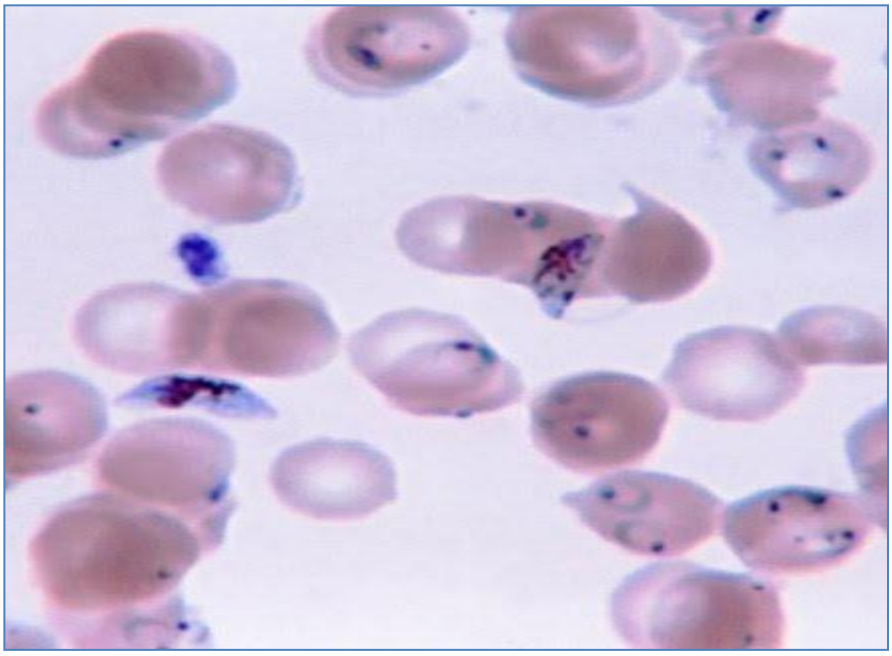


Figure 1.4: *Plasmodium falciparum*, thin blood smear showing trophozoites (ring stages) in the erythrocytes.

From the thick film, an experienced microscopist can detect parasite levels (or parasitemia) down to as low as 0.0000001% of red blood cells. Diagnosis of species can be difficult because the early trophozoites ("ring form") of all four species look identical and it is never possible to diagnose species on the basis of a single ring form; species identification is always based on several trophozoites (Figure 1.4). One important thing to note is that *P. malariae* and *P. knowlesi* (which is the most common cause of malaria in South-east Asia) look very similar under the microscope. However, *P. knowlesi* parasitemia increases very fast and causes more severe disease than *P. malariae*, so it is important to identify and treat infections quickly. Therefore modern methods such as PCR or monoclonal antibody panels that can distinguish between the *Plasmodium* species should be used in this part of the world. (McCutchan *et al.* 2008).

Taxonomical description of malarial parasites

Malaria is caused by a parasite called *Plasmodium*, which is transmitted via the bites of infected mosquitoes. In the human body, the parasites multiply in the liver, and then infect red blood cells. Malaria is a common vector borne disease in Bangladesh. Malaria has a long history in Indian subcontinent. It is now endemic in 13 districts of Bangladesh.

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- *Plasmodium falciparum*
Plasmodium vivax
Less Wide Spread Species- *Plasmodium malariae*
Plasmodium ovale
Plasmodium knowlesi

In Bangladesh, *P. falciparum* and *P. vivax* are the two most available species. Among these two species most deadly is *P. falciparum*.

General description of *Plasmodium falciparum*

The structural character of the parasite can be studied in stained preparations (Giemsa stained) (Figure 1.1). 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)

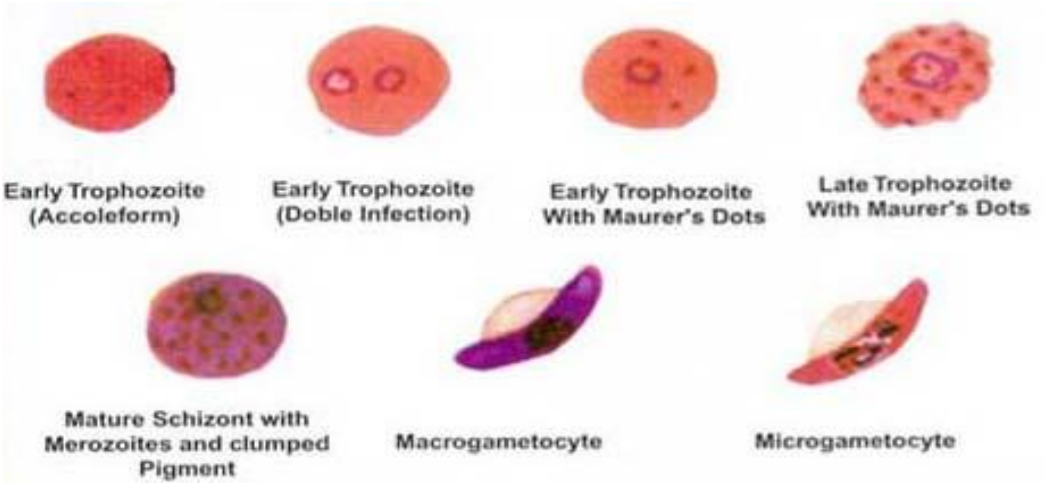


Figure 1.5: Different stages of *Plasmodium falciparum* in human blood.

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.* 2006).

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*. 2006) 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 2009). A brief life cycle of *P. facliparum* is provided in Figure 1.6.

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).

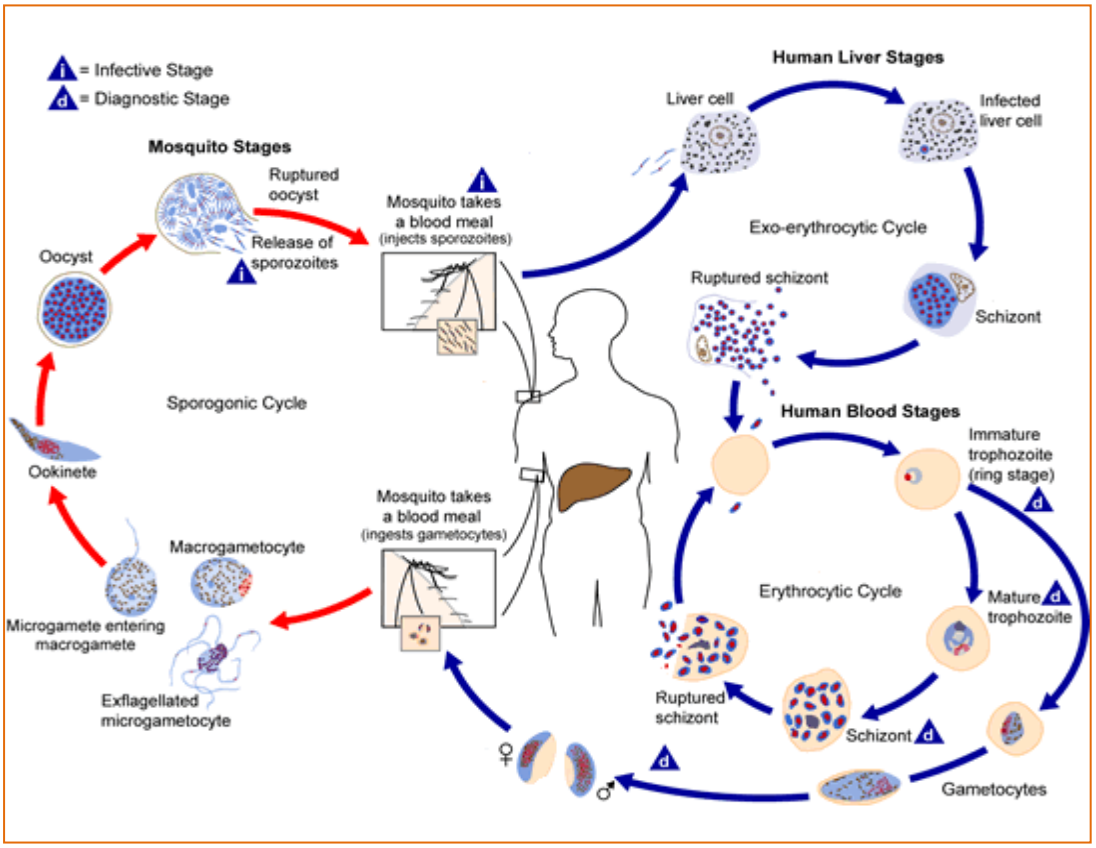


Figure 1.6: Life cycle of *Plasmodium falciparum*.

Signs and Symptoms of Malaria

Symptoms of malaria include fever, shivering, arthralgia (joint pain), vomiting, anemia (caused by hemolysis), hemoglobinuria, retinal damage, and convulsions (Figure 1.7). 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.

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.

Renal failure is a feature of black water fever, where hemoglobin from lysed red blood cells leaks into the urine. Severe malaria can progress extremely rapidly and cause death within hours or days. In the most severe cases of the disease, fatality rates can exceed 20%, even with intensive care and treatment. In endemic areas, treatment is often less satisfactory and the overall fatality rate for all cases of malaria can be as high as one in ten. Over the longer term, developmental impairments have been documented in children who have suffered episodes of severe malaria.

Chronic malaria can be seen in both *P. vivax* and *P. ovale*, but not in the case of *P. falciparum*. Here, the disease can relapse months or years after exposure, due to the presence of latent parasites stages in the liver. Describing a case of malaria as cured by observing the disappearance of parasites from the bloodstream can, therefore, be misleading. The longest incubation period was reported for a *P. vivax* infection, which

was 30 years (White 2003). Approximately one in five of *P. vivax* malaria cases in temperate areas involve overwintering by hypnozoites (i.e., relapses begin the year after the mosquito bite).

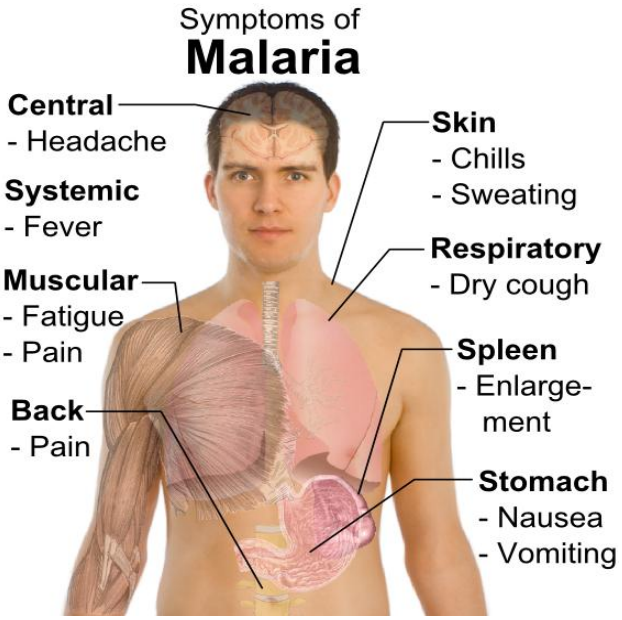


Figure 1.7: Symptoms of Malaria.

CHAPTER 2

REVIEW OF LITERATURE

Malaria Epidemiology

Malaria is a major health burden that is widespread throughout tropical and subtropical regions of the world. A lot of works were conducted regarding the prevalence of malaria, prevalence of *Plasmodium* which is responsible for the causation of malaria, distribution and abundance of *Plasmodium* carriers (vector species), collection techniques of *Anopheles* mosquitoes and determination of the *Plasmodium* infection status of anophelines and KAP studies. These studies are vast source of knowledge and reviewing of these literatures will enable us to know about many aspects that are relevant to the present study.

Learmonth (1957) stated that, the physical and cultural environments in Bangladesh permit the survival of at least two malarial parasites. He confirmed that Bangladesh should be considered a malaria-endemic area, and some parts of the country designated as hyperendemic.

Khan and Talibi (1972) carried out a longitudinal epidemiological study in Khagrachari of Chittagong Hill Tacts. They observed the transmission of malaria with two peaks, one in the pre-monsoon period, the other in the pre-winter period. New infections occurred in 51.7% of the infants surveyed who were 2 months old or under when first examined and there were frequent super-infections. The maternal immunity passively transferred to the infants did not play a significant role in restricting the development of the asexual erythrocytic stage or that of gametocytes, as indicated by the fact that the average parasite density and the gametocyte counts were highest in the 0-2-month age group and declined with increasing age. The found *Anopheles minimus* as the principal vector, but *An. leucosphyrous* was also found to be positive for *Plasmodium* sporozoite.

Dutt *et al.* (1980) stated that a malaria control program was started in India immediately after independence in 1947. Spraying with insecticides was the main control activity. Although, the disease was largely controlled by 1965, resurgence took place from several pockets. They demonstrated that monsoon rains, higher humidity, vegetation, tribal habitats and rice-cultivation have definitive associations with the disease in those two states.

Rosenberg and Maheswary (1982) studied on the forest malaria in Bangladesh. The malaria status of an isolated, forest-dwelling community was studied for 21 consecutive months. Blood examination, indirect hemagglutination testing, and detailed histories were used. Nearly 88% were found patent for *Plasmodium falciparum* and 70% for *P. vivax* at least once during the study. The population displayed characteristics of intense, annual transmission; asymptomatic patent infections, low trophozoite and gametocyte densities, and increasing antibody and decreasing parasite prevalence with advancing age. Prevalence and mean titer of antibody increased in each age group during the 7-month transmission season. Chloroquine-resistant *P. falciparum* was also demonstrated epidemiologically in this study.

Paul (1984) studied on history of malaria epidemiology in Bangladesh. He described that, the Government of Pakistan, initiates a Malaria Eradication Programme (MEP) in 1961 and it was successful since 1971 in controlling malaria where the rate of death due to Malaria drops down to 4.22 per 100000. But after the Liberation war, the MEP was interrupted. Due to this, the death rate rose to a peak of 60.44 in 1976.. The increase of malaria prevalance was particularly observable in the central and eastern districts of Bangladesh. The districts of Chittagong, the Chittagong Hill Tracts, and Sylhet were found highly prevalent in that study. The least incidence of malaria was in the western half of the country, where the eradication program had been initiated. Physical factors seemed to be more influential than manmade ones in the net change of incidence.

Beljaev *et al.* (1987) carried out a study on detection of malaria at primary health centers of India. He showed that annual *P. vivax* incidence was dropped 9.93 in 1976 to 2.06 in 1983 and annual *P. falciparum* incidence decline from 1.31 in 1976 to 0.76 in 1977.

Rodriguez-Del Valle *et al.* (1991) detected antigens and antibodies for *P. falciparum* malaria in the urine of humans. Humans infected with *P. falciparum* frequently have elevated levels of proteins in their urine, but it was unclear if any of these proteins were parasite antigens or antimalarial antibodies. The detection of malarial antigens and antibodies in urine may lead to a new approach for the diagnosis of malaria.

Bangali (1996) conducted a study on the perspective of border malaria in Bangladesh. A record was made during 1992-95 period of all malaria cases and control of all endemic border areas of Bangladesh.

Hossain *et al.* (1996) conducted a study to understand clinical pattern of malaria cases in Chittagong Medical College Hospital. They recruited *P. falciparum* malaria cases most of whom (80.55%) had a history of recent travel to hilly/remorte areas. Of them, 64.80% had severe malaria, 28.24% uncomplicated malaria and 6.95% treatment failure were also observed.

Noedl *et al.* (2003) studied efficacy of antimalaria drug in Bangladesh. They found high prevalence of in vitro mefloquine resistance in Bangladesh suggested that close surveillance is necessary to delay widespread multidrug resistant problems in the area.

Singh *et al.* (2003) carried out a parasitologic and entomologic cross-sectional survey in Madhya Pradesh in central India. During the survey period (December 1998) they found more than 70% of the fever cases were actually caused by malaria and of those 87% malaria cases was caused by *P. falciparum*. During follow up survey, in November 1999, 58% of the inhabitants were infected by malaria, with 80% of these cases caused by *P. falciparum*. They found *An. culicifacies* as the dominant mosquito species during the surveys.

Dev *et al.* in 2004 conducted fever surveys in several districts of the Indian state of Assam to ascertain the prevalence of malaria in relation to vector abundance, entomologic inoculation rates (EIRs) and geographic location of human settlements. *An. minimus* were incriminated, but their relative abundance and biting rates varied among districts, and no significant correlation was observed. *Plasmodium falciparum* was the predominant parasite species except in two districts where *P. vivax* was the main parasite. The EIRs per person/night were 0.46−0.71 in *P. falciparum*-predominant areas and 0.12 in the district where *P. vivax* predominated. Malaria cases were detected in all months of the year but peaked during May−June, which corresponded to the months of heavy rainfall. These were also the months with highest incidence of infection with *P. falciparum.* Malaria cases were observed in all age groups of both sexes, and there was clustering of cases in villages near the vector-breeding habitat (perennial seepage streams), and foothill villages. The data presented

by them are indicative of low-to-moderate levels of malaria transmission by *An. minimus*, and would be of value for developing future intervention strategies.

Keiser *et al.* (2005) assessed the impact of irrigation and dam building on malaria prevalence or incidence, stratified by the World Health Organization's sub-regions of the world, and link these studies with the latest statistics on disability adjusted life years, irrigated agriculture, and large dams. They also estimated the population at risk due to proximity to irrigation schemes and large dam reservoirs. In WHO sub-regions 1 and 2, which have 87.9% of the current global malaria burden, only 9.4 million people were estimated to live near large dams and irrigation schemes. In contrast, the remaining sub-regions (this region includes Kaptai dam of Bangladesh) concentrate an estimated 15.3 million people near large dams and up to 845 million near irrigation sites, while here only 12.1% of the global malaria burden was concentrated. They showed that individual water project triggers an increase in malaria transmission depends on the contextual determinants of malaria, including the epidemiologic setting, socioeconomic factors, vector management, and health seeking behavior. They concluded that in unstable malaria endemic areas, integrated malaria control measures, coupled with sound water management, were mandatory to mitigate the current burden of malaria in locations near irrigation or dam sites.

Rahman *et al.* (2006) correlated malaria epidemiologic data with satellite-based vegetation health (VH) indices to investigate if they can be used as proxy for monitoring malaria epidemics in Bangladesh by. The VH indices were represented by the vegetation condition index (VCI) and the temperature condition index (TCI). The VCI and TCI estimate moisture and thermal conditions, respectively. Sensitivity of VCI and TCI was assessed using correlation and regression analysis. They found that during cooler months (November–March) when mosquitoes are less active, the correlation was low. But it increased considerably during the warm and wet season (April–October), reaching 0.7 for the TCI in early October and −0.66 for the VCI in mid September.

Singh *et al.* (2006) carried out a longitudinal study on malaria in the Panna district in central India. Both *Plasmodium vivax* and *P. falciparum* were prevalent; however, the risk of *P. falciparum* malaria was 31.6% which is four times higher compared with

that of *P. vivax* malaria (7.8%). Also in this study *An. culicifacies* was found the dominant mosquito species.

Oduro *et al.* (2007) studied severe malaria caused by the *P. falciparum* in young children of the Kassena-Nankana district of northern Ghana. Of the total children screened, 45.2% (868/1921) satisfied the criteria for severe malaria. Estimated incidence of severe malaria was 3.4% (range: 0.4–8.3%) cases per year. The disease incidence was seasonal: 560 cases per year, of which 70.4% occurred during the wet season (June-October). The main manifestations were severe anaemia (36.5%); prolonged or multiple convulsions (21.6%); respiratory distress (24.4%) and cerebral malaria (5.4%). Others were hyperpyrexia (11.1%); hyperparasitaemia (18.5%); hyperlactaemia (33.4%); and hypoglycaemia (3.2%). The frequency of severe anaemia was 39.8% in children of six to 24 months of age and 25.9% in children of 25–60 months of age. More children (8.7%) in the 25–60 months age group had cerebral malaria compared with 4.4% in the 6–24 months age group. The overall case fatality ratio was 3.5%. Cerebral malaria and hyperlactataemia were the significant risk factors associated with death. Severe anaemia, though a major presentation, was not significantly associated with risk of death.

To clarify mechanisms linking deforestation, anopheline ecology and malaria epidemiology, Yasuoka and Levins (2007) analyzed 60 examples of changes in anopheline ecology and malaria incidence as a consequence of deforestation and agricultural development. The deforestation projects were classified based on subsequent land use and were reviewed in terms of their impact on anopheline density and malaria incidence. To further examine different anopheline responses to land transformation, two major ecological characteristics of 31 anopheline species were tested for their associations with changes in their densities and malaria incidence. Although niche width of anopheline species was not associated with density changes, sun preference was significantly associated with an increase in density. Their study suggested that the possibility of predicting potential impacts of future deforestation on vector density by using information on types of planned agricultural development and the ecology of local anopheline species.

Yasinzai and Kakarsulemankhel (2008) made a study to investigate the incidence of malarial infections in human population in Zhob, Balochistan, Pakistan. They

identified the malarial parasites from the blood slides collected from the suspected patients where 41.8% found to be positive for malaria parasite in blood smear slides. From the positive cases 51.8% were identified as *P. vivax* infection and 48.1% cases with *P. falciparum*. They did not find any *P. malariae* and *P. ovale* case in their study.

Maude *et al.* in 2008 conducted a descriptive study on malaria prevalence in the Southeast Bangladesh. From the blood samples they collected, they found 11.6% of samples were positive for *P. falciparum* and 4.9% for *P. vivax* i.e. 70.3% of all malaria cases were caused by *P. falciparum*. Mixed infection with both species was found in 0.01% of blood samples screened.

In first ever national malaria prevalence survey in Bangladesh, Haque *et al.* (2009a) found that among these 13 malaria endemic districts, the overall malaria prevalence rate was 3.1%. The overall prevalence of *P. falciparum* was 2.73% and that for *P. vivax* was 0.16%. Mixed infection of *P. falciparum* / *P. vivax* had a prevalence of 0.19%. The proportion of *P. falciparum* in all parasite positive samples was 88.6%. *P. vivax* and mixed infection had been detected in 5.2% and 6.25% of infections, respectively. In the same study found 11% overall malaria prevalence in Chittagong Hill Tracts.

A malaria baseline socioeconomic and prevalence survey was conducted by Ahmed *et al.* (2009) in Bangladesh aiming to the study of the knowledge/awareness of the respondents on malaria, their health-seeking behaviour relevant to malaria, study the possession and use patterns of bed net, the prevalence of malarial infection including species-specific prevalence (*P. vivax and P. falciparum*) and its distribution. The key findings of their survey were the general health awareness of the respondents was high, those from south/south-east (SSE) area trailed behind those from the north/north-east (NNE) area, albeit marginally, however, the knowledge on causation, transmission, prevention and control of malaria was not comprehensive, education, SES and gender were important determinants in shaping these different aspects of malaria, marginal differences were observed with respect to the two areas, the possession of at least one bed nets by the households was almost universal, the total number varying according to asset quintiles; however, insecticide treatment of bed nets was poor, major proportion of patients with malaria-like febrile illness either did

not seek any treatment or practiced self-treatment only, of those who sought treatment, majority went to the informal allopathic providers; gender and SES gradient disfavouring women and poorest was observed, for majority of the patients, treatment was delayed beyond 24 hours, the cost for malaria-like illness is quite high, especially in the NNE area.

Haque *et al.* (2009b) conducted a study in a highland area of Bangladesh to detect the clusters of malaria and to identify the geographic risk factors. They found that the average malaria prevalence in the District was 15.47% (n = 750). Their SaTScan detected five geographic clusters of malaria in Khagrachari district, one of which was highly significant (p = 0.001). They concluded that malaria cases were significally associated with proximity to water bodies and forests.

Rahman *et al.* (2010) reported a *P. malariae* case in Bangladesh. They described a 32-year-old Bangladeshi male presenting with severe malaria caused by a mono-infection with *P. malariae*. Rosetting of infected and uninfected erythrocytes, a putative virulence factor in *falciparum* malaria, was observed in the blood slide. Severe disease caused by *P. malariae* is extremely rare. The patient made a rapid recovery with intravenous quinine treatment.

The presence of *P. ovale* has never been reported from Bangladesh until 2010 (Fuehrer *et al.* 2010). They used a genus and species-specific nested polymerase chain reaction, targeting highly conserved regions of the small subunit ribosomal RNA (SSU rRNA) gene, to investigate the presence of malaria parasites in a total number of 379 patient samples in a survey of patients with febrile illnesses in the Chittagong Hill Tracts in Southeastern Bangladesh. They identified the first cases of *P. ovale* in Bangladesh. They were confirmed by sequence analysis; 189 of 379 samples (49.9%; 95% confidence interval = 44.9-54.9%) were positive for *Plasmodium* sp. by PCR. *P. falciparum* monoinfections accounted for 68.3% (61.3-74.5%), followed by *P. vivax* (15.3%; 10.9-21.2%), *P. malariae* (1.6%; 0.5-4.6%), *P. ovale* (1.6%; 0.5-4.6%), and mixed infections (13.2%; 9.1-18.8%). They did not found any evidence of *P. knowlesi* in this region.

Yusuf *et al.* (2010) studied fever cases among the children less than five years of age were examined by in Nigeria. About 16% of children reported having fever in the two weeks preceding the survey. The prevalence of fever was highest among children from the poorest households (17%), compared to 15.8% among the middle households and lowest among the wealthiest (13%). They concluded that while, overall bednet possession was low and less fever was reported in households that possessed bednets.

Haque *et al.* (2011) studied malaria prevalence, risk factors and spatial distribution in a hilly forest area of Bangladesh. In this study a 2-stage cluster sampling technique was used to sample 1,400 of 5,322 (26.3%) households in Rajasthali, and screened using a rapid diagnostic test (Falcivax). Overall malaria prevalence was 11.5%. The proportions of *P. falciparum*, *P. vivax* and infection with both species were 93.2%, 1.9% and 5.0%, respectively. Univariate, multivariate logistic regression and spatial cluster analyses were performed separately. Sex, age, number of bed nets, forest cover, altitude and household density were potential risk factors. A statistically significant malaria cluster was identified. Significant differences among risk factors were observed between cluster and non-cluster areas. They conclude that malaria has significantly decreased within 2 years after onset of intervention program. Both aspects of the physical and social environment, as well as demographic characteristics are associated with spatial heterogeneity of risk. The ability to identify and locate these areas can provide a strategy for targeting interventions during initial stages of intervention programs. However, in high risk clusters of transmission, even extensive coverage by current programs can leave transmission ongoing at reduced levels.

Kumar *et al.* (2012) compiled a review of malaria prevalence in India. They found poor access to health care went hand in hand with poor reporting from some of the same areas, combining to possibly distort disease prevalence and death rom malaria in some parts of India. They also mentioned that corrections were underway in the form of increased resources for disease control, greater engagement of village-level health workers for early diagnosis and treatment, and possibly new public-private partnerships activities accompanying traditional national malaria control programs in the most severely affected areas of India.

Knowledge attitude and practice (KAP) on malaria

A review and summary of the social and behavioral risk factors that was proved to be associated with malaria in Southeast Asian countries was conducted by Elsie and Funglada (1994). They demonstrated that these risk factors 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.

Adongo *et al.* (2005) conducted a survey to evaluate the effect of local community knowledge about malaria affects insecticide treated net use in northern Ghana. 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 towards malaria prevention measure was conducted by Erhun *et al*. (2006) in Nigeria. Their analysis of "what respondents will do first" during malaria attack showed that 35.5%, 0.9% and 13.4% of respondents will use synthetic anti-malarials, consult a herbalist and use local herb, respectively, while 27.3%, 1.7% and

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.

Soan and Chand (2006) conducted a KAP program in tribal population in India. Results from that study 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.

Keating *et al*. (2008) evaluated a malaria related KAP survey in Haiti. The two-stage cluster survey 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 precention and treatment options.

A two-stage cluster sampling technique was used by Ahmed *et al*. (2009) to perceive Knowledge on the transmission, prevention and treatment of malaria among two endemic populations of Bangladesh 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.

Haque *et al*. (2010) reported the progress and challenges in malaria control strategies in Bangladesh. This study 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.

Molecular diagnosis of malaria

Prompt and accurate diagnosis of malaria is needed for implementation of appropriate treatment to reduce associated morbidity and mortality. 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 were designed for specific amplification by the Polymerase Chain Reaction (PCR), to detect each malaria species. Review of literature about diagnosis of Malaria will enable us to know about many aspects that are relevant to this study.

Snounou *et al.* (1993a) 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. DNA equivalent to 5 µL of blood was sufficient for the detection of each of the 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. A high proportion of mixed species infections were brought to light by the PCR assay.

Snounou *et al.* (1993b) showed that the use of nested primers allows the detection and identification of very low numbers of the four human malaria parasites, without the requirement for further blotting and hybridisation of the PCR amplification product. The addition of a PCR amplification step, was justified because of the resulting high sensitivity of detection.

Das *et al.* (1995) worked on a study to investigate the species-specific 18S rRNA gene amplification for the detection of *P. falciparum* and *P. vivax* malaria parasites. Based on the sequence diversity of the *Plasmodium* 18S ribosomal RNA (rRNA), they designed oligonucleotide primers for polymerase chain reaction (PCR) to yield different size fragments for *P. falciparum* and *P. vivax*. The primers for the PCR procedure were chosen such that the 5' primer was Plasmodium-conserved while the 3' primers were species-specific. Using primer cocktails and cloned plasmid DNAs containing the 18S rRNA genes or parasite genomic DNA as targets, we show that the PCR procedure yields 1.4-kb and 0.5-kb DNA fragments for *P. falciparum* and *P. vivax*, respectively. Limited field testing of this procedure demonstrated the utility of a ribosomal gene based species-specific malaria diagnosis.

Singh *et al*. (1996), revealed on their study about a detection of malaria in Malaysia by nested polymerase chain reaction amplification of dried blood spots on filter papers. A modified nested polymerase chain reaction (PCR) method for detection of *Plasmodium falciparum, P. vivax* and *P. malariae* was combined with a simple blood collection and deoxyribonucleic acid (DNA) extraction method and evaluated 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. The simple Chelex method was used to prepare DNA templates for the nested PCR assay. 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. The nested PCR method, when coupled with simple dried blood spot sampling, is a useful tool for collecting accurate malaria epidemiological data, particularly in remote regions of the world.

Zalis *et al.* (1996) attempted for malaria diagnosis: standardization of a polymerase chain reaction for the detection of *Plasmodium falciparum* parasites in individuals with low-grade parasitemia. They described a highly sensitive methodology for malaria diagnosis using a nested PCR method based on amplification of the *p126 P. falciparum* gene detected by simple ethidium bromide staining. The *P. falciparum* Palo Alto strain (culture samples) was serially diluted in blood from an uninfected donor to a final level of parasitemia corresponding to 10–8% and was processed for PCR amplification. In each of these dilutions a parasitological examination was performed to compare the sensitivity with that of PCR amplification. Blood samples (field samples) were obtained from 51 malarious patients with positive thick blood smears (TBS) who were living in endemic regions of the Brazilian Amazon. They corresponded to 42 *P. falciparum* and 9 *P. vivax* cases, with parasitemia levels ranging from only 16 to 20,200 parasites/ml for *P. falciparum disease* and from 114 to 11,000 parasites/ml for *P. vivax* malaria. They demonstrated that the use of nested PCR allowed the detection of 0.005 parasites/ml without the use of any radioactive material.

Kawamoto *et al.* (1996) evaluated the sequence variation in the 18S rRNA gene, a target for PCR-Based Malaria Diagnosis, in *Plasmodium ovale* from Southern Vietnam. Field surveys of malaria were performed in southern Vietnam by using an

acridine orange staining method for rapid diagnosis and a PCR-based, microtiter plate hybridization method for accurate diagnosis. A total of three patients of *Plasmodium ovale* infection were detected, but PCR-amplified DNA of the *P. ovale* isolates from two of the patients did not hybridize with the *P. ovale*-specific probe. Analysis of the target sequence in the 18S rRNA gene indicated that in the DNA of isolates from both patients three nucleotides in the probe region from the typical *P. ovale* sequence were different, with deletions of two nucleotides and the substitution of one nucleotide..

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 *Plasmodium falciparum* and two *Plasmodium vivax* parasites/microliter of whole blood by nested PCR without compromising the simplicity of specimen collection or DNA extraction.

Tahar and Basco (1997) made an extensive study on the detection of *Plasmodium ovale* malaria parasites by species-specific 18S rRNA gene amplification. A polymerase chain reaction (PCR) assay was developed for the specific detection of *Plasmodium ovale*, one of the four malaria parasites that infect humans. On the basis of sequence variation of the *Plasmodium* 18S ribosomal RNA (rRNA) gene, oligonucleotide primers for PCR were designed to amplify various fragments of the *P. ovale* gene. Using a recombinant plasmid with the complete *P. ovale* 18S rRNA gene as target, 59 primer combinations were tested so that at least one of the pairs was species-specific while the other primer was either genus conserved or *P. ovale* species-specific. Three primer pairs yielding DNA fragments at stringent conditions were further tested against genomic DNA of four human malaria species. This approach yielded *P. ovale* species-specific primer pairs that may be useful for further field testing.

Singh *et al*. (1999) worked on a genus and species-specific nested polymerase chain reaction malaria detection assay for epidemiologic studies. The assay was evaluated on fingerprick blood samples collected on filter paper from 129 individuals living in a malaria-endemic area in Malaysia. Malaria prevalence by genus-specific nested PCR

was 35.6% (46 of 129) compared with 28.7% (37 of 129) by microscopy. The nested PCR detected seven more malaria samples than microscopy in the first round of microscopic examination, malaria in three microscopically negative samples, six double infections identified as single infections by microscopy and one triple infection identified as a double infection by microscopy. The nested PCR assay described is a sensitive technique for collecting accurate malaria epidemiologic data. When coupled with simple blood spot sampling, it is particularly useful for screening communities in remote regions of the world.

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 *Plasmodium 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.

Toma *et al.* (1999) carried out a study on *Plasmodium ovale* was demonstrated in 5 out of 143 inhabitants in a village in Lao PDR by blood microscopy and PCR assay. Although the specimen confirmed to be positive for *P. ovale* by microscopical examination was only one, the target sequences in the 18S rRNA genes of malaria parasite detected in all of the five cases were consisted with those of *P. ovale* by the PCR assay. This was the first report concerning the presence of so many cases with *P. ovale* infection in Lao PDR.

Zakeri *et al.* (2002) detected of malaria parasites by nested PCR in south-eastern, Iran: Evidence of highly mixed infections in Chahbahar district. To evaluate these criteria and in a comparative study, blood specimens were collected from 120

volunteers seeking care at the Malaria Health Center in Chahbahar district. One hundred and seven out of 120 Giemsa-stained slides were positive for malaria parasites by microscopy. Eighty-four (70%) and 20 (16.7%) were identified as having only *Plasmodium vivax* and *Plasmodium falciparum* infections, respectively, while only 3 (2.5%) were interpreted as having mixed *P. vivax-P. falciparum* infections. The target DNA sequence of the 18S small sub-unit ribosomal RNA (ssrRNA) gene was amplified by Polymerase Chain Reaction (PCR) and used for the diagnosis of malaria in south-eastern Iran. One hundred twenty blood samples were submitted and the results were compared to those of routine microscopy. The sensitivity of PCR for detection of *P. vivax* and *P. falciparum* malaria was higher than that of microscopy: nested PCR detected 31 more mixed infections than microscopy and parasite positive reactions in 9 out of the 13 microscopically negative samples. These results suggest that, in places where transmission of both *P. vivax* and *P. falciparum* occurs, nested PCR detection of malaria parasites can be a very useful complement to microscopical diagnosis.

Perandin *et al.* (2003) identified *Plasmodium falciparum, P. vivax, P. ovale* and *P. malariae* and mixed infection in patients with imported malaria in Italy. The species-specific nested-PCR previously described by Snounou and others for detecting the four parasite species that cause human malaria is evaluated in the current study testing 230 blood samples. The results are compared with those obtained by microscopy and, for 101 samples out of 230, with those previously obtained by a genus-specific PCR based method followed by species-specific Southern-blot hybridization. All blood specimens were obtained from patients (127 foreigners and 103 Italians) with a suspect clinical diagnosis of imported malaria in Italy: 76 were positive by microscopy and 83 were positive by nested-PCR. The last method also revealed 10 double infections (8 foreigners and 2 Italians) which were not identified by microscopy or by pg-PCR with species-specific Southern-blot hybridization. Fifty-four out of 83 positive samples tested by nested-PCR were submitted to genomic sequence analysis, which confirmed the presence of DNA region portion encoding the 18S rRNA corresponding to the *Plasmodium* species identified by nested-PCR. These results demonstrated that the nested-PCR assay surpasses microscopy and genus-specific -PCR with species-specific Southern-blot hybridization, both in sensitivity and in diagnostic accuracy. Moreover, it was quicker because it requires no further

blotting or hybridization of PCR amplification products. This method also offered a clear advantage in the detection of mixed infections, which was important not only for successful medical treatment but also for the study of malaria epidemiology. Finally, their study also highlights the value of genomic sequence analysis for validating PCR results.

Siribal *et al.* (2004) worked on identification of human malaria parasites and detection of mixed infection in Thai patients by nested PCR. The species-specific nested PCR for detecting the four species of human malaria parasites, is evaluated in the current study 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 *Plasmodium 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 *Plasmodium 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.

Di Santi *et al.* (2004) studied on PCR – based diagnosis to evaluate the performance of malaria reference centers. Although the Giemsa-stained thick blood smear (GTS) remains the gold standard for the diagnosis of malaria, molecular methods are more sensitive and specific to detect parasites and can be used at reference centers to evaluate the performance of microscopy. The description of the *Plasmodium falciparum*, *P. vivax*, *P. malariae* and *P. ovale* ssrRNA gene sequences allowed the development of a polymerase chain reaction (PCR) that had been used to differentiate the four species. The objective of this study was to determine *Plasmodium* species

through PCR in 190 positive smears from patients in order to verify the quality of diagnosis at SUCEN's Malaria Laboratory. Considering only the 131 positive results in both techniques, GTS detected 4.6% of mixed and 3.1% of *P. malariae* infections whereas PCR identified 19.1% and 13.8%, respectively.

Hay *et al.* (2004) published a review on the past, present, and future global distribution and population at risk of malaria. They reviewed the spatial distribution of malaria and human populations at risk through time. They also considered the global malaria control programs from 1900 to 2002 and the consequences of changing global population at risk of malaria in their review article.

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.

A study was conducted by Ebrahimzadeh *et al.* (2007), high rate of detection of mixed infections of *Plasmodium vivax* and *Plasmodium falciparum* in South-East of Iran, using nested PCR. Blood specimens were collected from 140 suspected volunteers. The Giemsa-stained slides examination and nested PCR for amplification of the *Plasmodium* small subunit ribosomal genes (ssrRNA) were utilized. The results demonstrated 118 out of 140 cases (84.3%) positive for malaria parasites, including 60.7%, 20.7% and 2.9% as having *Plasmodium vivax* (*P.v*), *Plasmodium falciparum*

(*P.f*) and mixed infections (*P.v+P.f*), respectively by microscopy. The nested PCR detected malaria parasites in 134 samples (94.3%), consisting of 51.4% *P.v*, 12.6% *P.f* and 29.3% mixed infections. The PCR analysis detected 37 cases of mixed infections more than that of the routine microscopy. These results suggested that there are a considerable number of cases with mixed infections in the study area that mainly remain undiagnosed by microscopy. It is also concluded that the nested PCR is a suitable complement to microscopy for accurate specific diagnosis of malaria species in field.

Mohapatra *et al.* (2008) screened a total of 1,995 fever cases resulted in 9 probable cases of *P. malariae* based on morphological identification in Chakma tribe people residing in 2 villages. Nested PCR confirmed the identity of all probable cases of *P. malariae* by producing diagnostic band of 144 bp. PCR method was able to detect mixed infection of *P. malariae* with *P. vivax* and with *P. falciparum*. *P. malariae* may have been present in Arunachal Pradesh but most probably is being misdiagnosed due to its close resemblance with *P. vivax*, especially in ring forms. Estimation of actual case load of *P. malariae* in north-east India was, therefore, important with accurate species identification using molecular methods.

Nizamuddin *et al.* (2009) attempted to identify the potential factors for malaria epidemic in forest hills in Bangladesh in their study. They estimated the correlation between various environmental factors that contribute to malaria transmission and showed the application of remote sensing data for improved predictions of malaria epidemics in Bandarban district of Bangladesh which had the highest frequency of malaria cases in the country.

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.

In a analytical study, Shahbazi *et al.* (2009) evaluated 100 positive blood samples for malaria by microscopic method (85 *Plasmodium vivax* and 15 *Plasmodium falciparum*) and 15 negative samples were collected from malarious areas of Iran and processed by Nested PCR to amplify the ssrRNA (small sub-unit ribosomal RNA). Except for one case, diagnosis either by microscopic or Nested-PCR was the same. The exception was for one case diagnosed *Plasmodium falciparum* by microscopic and in agreement method whiteas mix infection (*Plasmodium vivax* and *Plasmodium falciparum*) by nested-PCR. Agreement coefficient of microscopic and nested-PCR methods was high.

Ahmed *et al.* (2009) conducted to evaluate the sensitivity and specificity of Immunochromatographic test (ICT) for antigen, using microscopy as the "gold standard" method for diagnosis of malaria. A total of 98 clinically suspected malaria patients and another 30 age and sex-matched healthy controls were included in this study. Thick and thin films were also prepared and examined under microscope as well as Immunochromatographic test (ICT) was performed for malaria antigen. Sensitivity and specificity of ICT for antigen were 93.22% and 94.87% respectively.

Andrade *et al.* (2010) carried out a study towards a precise test for malaria diagnosis in the Brazilian Amazon: comparison among field microscopy, a rapid diagnostic test, nested PCR, and a computational expert system based on artificial neural networks nested PCR was shown to be the gold standard for diagnosis of both symptomatic and asymptomatic malaria because it detected the major number of cases and presented

the maximum specificity. Surprisingly, the RDT was superior to microscopy in the diagnosis of cases with low parasitaemia. Nevertheless, RDT could not discriminate the *Plasmodium* species in 12 cases of mixed infections (*Plasmodium vivax* + *Plasmodium falciparum*). Moreover, the microscopy presented low performance in the detection of asymptomatic cases (61.25% of correct diagnoses). The MalDANN system using epidemiological data was worse that the light microscopy (56% of correct diagnoses). However, when information regarding plasma levels of interleukin-10 and interferon-gamma were inputted, the MalDANN performance sensibly increased (80% correct diagnoses).

Parija (2010) concluded that PCR is valuable as a confirmatory test and implementation should be considered by reference laboratories and worldwide with adequate laboratory infrastructure to perform molecular procedures.

To *et al.* (2010) carried out a study on the complication of Corticosteroid Treatment by Acute *Plasmodium malariae* infection confirmed by Small-Subunit rRNA Sequencing. They reported a case of acute *Plasmodium malariae* infection complicating corticosteroid treatment for membranoproliferative glomerulonephritis in a patient from an area where *P. malariae* infection was not endemic. A peripheral blood smear showed typical band-form trophozoites compatible with *P. malariae* or *Plasmodium knowlesi*. SSU rRNA sequencing confirmed the identity to be *P. malariae*.

Al-Mekhlafi *et al.* (2010) studied the molecular epidemiology of *Plasmodium* species prevalent in Yemen based on 18 s rRNA. This study was conducted to investigate the distribution of the *Plasmodium* species based on the molecular detection and to study the molecular phylogeny of these parasites. Blood samples from 511 febrile patients were collected and a partial region of the 18 s ribosomal RNA (18 s rRNA) gene was amplified using nested PCR. From the 86 positive blood samples, 13 *Plasmodium falciparum* and 4 *Plasmodium vivax* were selected and underwent cloning and, subsequently, sequencing and the sequences were subjected to phylogenetic analysis using the neighbor-joining and maximum parsimony methods. Malaria was detected by PCR in 86 samples (16.8%). The majority of the single infections were caused by *P. falciparum* (80.3%), followed by *P. vivax* (5.8%). Mixed infection rates of *P. falciparum + P. vivax and P. falciparum + P. malariae* were 11.6% and 2.3%,

respectively. All *P. falciparum* isolates were grouped with the strain 3D7, while *P. vivax* isolates were grouped with the strain Salvador1. Phylogenetic trees based on 18s rRNA placed the *P. falciparum* isolates into three sub-clusters and *P. vivax* into one cluster. Sequence alignment analysis showed 5-14.8% SNP in the partial sequences of the 18 s rRNA of *P. falciparum*. Although *P. falciparum* was predominant, *P. vivax, P. malariae* and mixed infections were found more prevalent than had been revealed by microscopy.

The presence of *P. ovale* has never been reported from Bangladesh before 2010. Fuehrer *et al.* (2010) used a genus and species-specific nested polymerase chain reaction, targeting highly conserved regions of the small subunit ribosomal RNA (SSU rRNA) gene, to investigate the presence of malaria parasites in a total number of 379 patient samples in a survey of patients with febrile illnesses in the Chittagong Hill Tracts in Southeastern Bangladesh. They identified the first cases of *P. ovale* in Bangladesh. They were confirmed by sequence analysis; 189 of 379 samples (49.9%; 95% confidence interval = 44.9-54.9%) were positive for *Plasmodium* sp. by PCR. *P. falciparum* monoinfections accounted for 68.3% (61.3-74.5%), followed by *P. vivax* (15.3%; 10.9-21.2%), *P. malariae* (1.6%; 0.5-4.6%), *P. ovale* (1.6%; 0.5-4.6%), and mixed infections (13.2%; 9.1-18.8%). They did not found any evidence of *P. knowlesi* in this region.

Demas *et al.* (2011) assessed the accurate and rapid diagnosis of malaria infections is crucial for implementing species-appropriate treatment and saving lives. Molecular diagnostic tools are the most accurate and sensitive method of detecting *Plasmodium*, differentiating between *Plasmodium* species, and detecting subclinical infections. Despite available whole-genome sequence data for *Plasmodium falciparum* and *P. vivax*, the majority of PCR-based methods still rely on the 18S rRNA gene targets. Historically, this gene had served as the best target for diagnostic assays.

Fontecha *et al.* (2012) made a study on comparison of molecular tests for the diagnosis of malaria in Honduras. Of the 69 microscopically positive *P. falciparum* samples, 68 were confirmed to be *P. falciparum*-positive by two of the molecular tests used. The one sample not detected as *P. falciparum* by any of the molecular tests was shown to be *P. vivax*-positive by a reference molecular test indicating a misdiagnosis by microscopy. The reference molecular test detected five cases of *P. vivax/P.*

falciparum mixed infections, which were not recognized by microscopy as mixed infections. Only two of these mixed infections were recognized by a multiplex test while a *P. vivax*-specific polymerase chain reaction (PCR) detected three of them. In addition, one of the day 28 samples, previously determined to be malaria negative by microscopy, was shown to be *P. vivax*-positive by three of the molecular tests specific for this parasite.

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. Methods used included Giemsa staining procedure for estimation of parasite densities and polymerase chain reaction (PCR) to detect the presence of malaria parasite in the whole blood. 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.

Notomi *et al.* (2000) described a novel DNA amplification method named loop mediated isothermal amplification (LAMP). In this method, a specific fragment of DNA can be amplified in heat block without a thermal cycler, which can be too expensive for field PCR. Four specially designed primers and two additional loop forming primers amplify the target DNA with the large fragment of *Bst* polymerase which has exclusive strand displacement activity in LAMP method. Immense amplification leads to accumulation of magnesium pyrophosphate, which turns the solution turbid, and can be visualized by the naked eye or measured with a turbidity meter.

Eventually, LAMP became of great interest to a number of investigators worldwide. Consequently, several diagnostic methods were implemented for protozoan parasite detection (Mori and Notomi 2009) as well as malaria (Abdul-Ghani *et al.* 2012).

Poon *et al.* (2006) first reported the use of LAMP for diagnosis of clinical malaria due to *P. falciparum* and later Han *et al.* (2007) implemented genus and species-specific LAMP for diagnosis of four *Plasmodium* species that infect humans using primers to an 18sRNA gene. Polley *et al.* (2010) reported better sensitivity of LAMP than earlier reports using primers designed targeting mitochondrial DNA.

Genetic diversity of *Plasmodium falciparum*

Merozoite surface proteins (MSP) are important vaccine candidate for malaria treatment. Among them MSP-1 is the most important one. But it shows polymorphisms which question the efficacy of vaccine. To make an effective vaccine it is needed to study the genetic diversity of the polymorphic allelic families. Only review of the published literature can provide the scope of the study.

Holder *et al.* (1992) worked on the structure, processing and function of the MSP-1 gene of malaria parasite. They found that, the protein in all malaria parasite species and the structural study on that gene revealed that parts of molecule are well conserved. A study on *P. falciparum* has shown that the protein is processed from the surface of the merozoite and helps in invasion of the red blood cells (RBC).

Analysis of sequence diversity in the *P. falciparum* MSP-1 gene was done by Miller *et al.* (1993). They aligned all published sequences, identified errors, re-sequenced a portion of one parasite clone and identified probable duplicate sequences of four pairs of parasite clones. The resultant sequences were displayed in a fashion that facilitates the study of variation and its potentially diverse origins.

Paul *et al.* (1995) studied the mating patterns in malaria parasite populations of rural Madang on the north coast of Papua New Guinea. They measured the oocyst heterozygosity and patterns of genetic disequilibrium using MSP-1, MSP-2 and GLURP as polymorphic markers from the samples of both human residents and blood fed mosquitoes by PCR using allele specific probes. Considerable allelic diversity was observed in parasite populations from the samples. There were 14 alleles identified for MSP-2, 9 alleles for MSP-1 and 9 alleles for GLURP in human samples, while 13 MSP-2 alleles, 8 MSP-1 alleles and 7 GLURP alleles in the oocysts. A comparison of

allele frequencies of the human and oocyst parasite populations was carried out independently for each locus: the human parasite populations were significantly different from the oocyst parasite populations for MSP-2 and GLURP ($P< 0.01$). They reported, mosquito and human parasite populations share most of the alleles but in different frequencies. The study provides 72% of the human population with multiple genotypes, whereas only 15% of the oocyst sample were heterozygous, taking all three loci into consideration. There was no linkage disequilibrium reported in that study.

Ntoumi *et al.* (1997) worked on the imbalanced distribution of *P. falciparum* MSP-1 genotypes related to sickle-cell trait in Gabon and Senegal in Africa. The polymorphic MSP-1 and MSP-2 loci were genotyped using a PCR-based methodology. In Senegalese carriers, age and hemoglobin type influenced differently the distribution of the three MSP-1 families and had an impact on distinct individual alleles, whereas the distribution of MSP-2 alleles was marginally affected. There was no influence of other genetic traits, including the HLA Bw53 genotype, or factors such as place of residence within the village. In a cohort of Gabonese schoolchildren in which the influence of age was abrogated, a similar imbalance in the MSP-1 allelic distribution but not of MSP-2 allelic distribution by hemoglobin type was observed.

In 1998, Paul *et al.* studied the transmission intensity and genetic diversity in *P. falciparum* on the Northwestern border of Thailand using MSP-1, MSP-2 and GLURP as the polymorphic markers by nested PCR based method. In that study, genetic analysis of the number of *P. falciparum* genotypes per infected person in regions of holoendemic and hyperendemic malaria produced fewer parasite genotypes per infected person. They didn't report any association between the number of genotypes per infected person with age or sex.

In 1998, Zwetyenga *et al.* concluded that, there is no influence of age on infection complexity. They analyzed the polymorphism in MSP-1, MSP-2 and GLURP gene by nested PCR based methodology. They reported 17 distinct MSP-1 alleles differing by size and/or allelic types, as many as 43 distinct MSP-2 alleles and 9 distinct GLURP alleles. On their study, there was no preferential carriage of any allelic family in any age group.

Allelic diversity at the MSP-1locus in natural *P. falciparum* populations was studied by Ferreira *et al.* (1998). They compared the patterns of allelic diversity at the MSP-1 locus in wild isolates from three epidemiologically distinct malaria-endemic areas: the hypoendemic southwestern Brazilian Amazon, the mesoendemic southern Vietnam and the holoendemic northern Tanzania. Fragments of the variable blocks 2, 4a, 4b and 6 or 10 of this single-copy gene were amplified by the polymerase chain reaction.

In 1999, Tanabe *et al.* published their work on the molecular epidemiology of MSP-1 of *P. falciparum*. They described the application of PCR based method in the molecular epidemiological aspect and the detectability and sensitivity of the PCR method.

A cohort study of *P. falciparum* diversity during the dry season in Ndiop, a Senegalese village with seasonal, mesoendemic area was done by Zwetyenga *et al.* (1999). They used MSP-1 and MSP-2 gene as polymorphic marker for the study using allele specific nested PCR method. They observed considerable fluctuations in the genotypes.

Snounou *et al.* (1999) studied on the allelic variation of *P. falciparum* populations in Thailand by nested PCR of three polymorphic regions of *P. falciparum* antigen genes: MSP-1, MSP-2 and GLURP. They observed highest degree of polymorphism in the MSP-2 gene and the Multiplicity of Infection (MOI) calculated with this marker was higher than that obtained using MSP-1 or GLURP alone. They observed 12, 17 and 10 variants for each polymorphic marker with different sized product.

Brockman *et al.* (1999) worked on parasite genotyping by PCR based method to distinguish recrudescent from newly acquired *P. falciparum* infections in Karen population resident on the northwestern border of Thailand where malaria transmission is low. *P. falciparum* infections were genotyped for allelic variation in three polymorphic antigen loci, MSP-1, MSP-2 and GLURP, before and after antimalarial treatment by nested PCR based methodology. Population genotype frequencies were measured to provide the baseline information to calculate the probability of a new infection with a different or the same genotype to the initial pretreatment isolate. Overall, 38% of the infections detected following treatment had an identical genotype before and up to 121 days after treatment. These post-treatment

genotypes were considered recrudescent because of the low (<5%) probability of repeated occurrence by chance in the same patient.

The genetic diversity displayed by *P. falciparum* field isolates, the occurrence of variant forms of the parasite at different frequencies and the multiplicity of the infections were studied by Haddad *et al.* (1999) in *P. falciparum* field isolates from Honduras, a region where its transmission is low and seasonal. Allelic diversity was analyzed in the highly polymorphic parasite genes encoding the MSP-1and MSP-2 and the GLURP by PCR. Gene polymorphism was also assessed in the EB200 region derived from the highly size polymorphic Pf332 gene.

Da Silveira *et al.* (1999) used PCR based method to type variable regions, blocks 2, 4, and 10, of the MSP-1 gene and to characterize major gene types (unique combinations of allelic types in variable blocks) in *P. falciparum* isolates collected across the Brazilian Amazon region. They also compared the antibody recognition of polymorphic (block 2), dimorphic (block 6), and conserved (block 3) regions of MSP-1 in Amazonian malaria patients and clinically immune Africans, using a panel of recombinant peptides.

In the work of Conway *et al.* (2001) nine microsatellite tri-nucleotide TAA(n) repeat loci (ta1, ta42, ta81, ta87, ta109, PfPK2, G377, Polyα and ARA2) and block 2 in the MSP-1 gene were genotyped in six population of Africa (located in The Gambia, Nigeria, Gabon, Sudan, Tanzania and South Africa), using PCR and sequence-specific oligonucleotide probing. They discriminated alleles by size of products amplified by PCR, with an observed number of distinct alleles per locus ranging from 7 (locus G377) to 22 (locus ta1). Overall, the microsatellite loci showed moderate inter-population variance in allele frequencies (mean F_{ST} value = 0.026), higher than that for block 2 of msp1. For all except one (G377) of these nine loci individually, the F_{ST} values exceeded that of block 2 and were highly significantly above zero ($P< 0.01$ for locus ARA2; $P < 0.001$ for each of the other seven loci).

Jordan *et al.* (2001) investigated population structure of *P. falciparum* isolates during an epidemic in southern Mauritania using thee nested PCR based methodology. They analyzed 386 samples for infection with *P. falciparum*, and 173 (45%) tested positive. The isolates were genotyped for three polymorphic genetic markers: MSP-1, MSP-2

and GLURP. The mean minimal number of clones in the non-endemic region around Aioun was 1.57, blood samples collected in the hypoendemic region around Kobeni showed multiple infections with an average of 2.34 clones ($P < 0.001$). In addition, clear differences between endemic regions were apparent in three of the investigated allelic groups: RO33 of the MSP1 gene and FC and Indochina of the MSP2 gene.

Peyerl-Hoffmann *et al.* (2001) investigated the genetic diversity of *P. falciparum* populations in areas with different transmission levels (holo- vs. mesoendemic) was investigated in Kabarole District, West Uganda. 225 samples positive for *P. falciparum* were analyzed by amplification of polymorphic regions and classified according to prevalence of allelic families. A large number of alleles were detected for each locus: 22 for MSP-1 block 2 and 24 for MSP-2 and, 175 (78%) of MSP-1 alleles and 143 (64%) of MSP-2 showed multiple infections within a range of 2±8 clones. Significant differences between holoendemic and mesoendemic areas in regards of population structure and number of multiclonal infections of *P. falciparum* were not apparent. However, a significant correlation between parasite density, selected MSP-2 loci and differences between parasite density in monoclonal vs. multiclonal infections occurred. Multiplicity of infection was found age-dependent.

Cowman *et al.* (2002) published a work entitled 'Functional analysis of *P. falciparum* merozoite antigens: implications for erythrocyte invasion and vaccine development'. The work described the functions of all MSPs and their importance in vaccine development of malaria.

Microsatellite characterization of *P. falciparum* from cerebral and uncomplicated malaria patients in southern Vietnam was studied by Ferreira *et al.* (2002) using 9 microsatellite polymorphic markers: *Polyα*, *TA81, TA1* and *TA87* , *2490* , *ARAII* , *PfG377* and *PfPK2* and *TA60*. Alleles were amplified by seminested PCR by using primers corresponding to conserved regions flanking the microsatellites. Temporal structure and linkage disequilibrium were also examined in that data set.

Aubouy *et al.* (2003) studied polymorphism in MSP-1 and MSP-2 genes of *P. falciparum* isolates from Gabon. They carried out the genotyping by nested PCR. 25 and 19 different alleles were obtained respectively for MSP-1 and MSP-2 loci, the RO33 family of MSP-1 being poorly polymorphic among the 52 Gabonese children

those were genotyped. All but two isolates were composed of more than one genotype and the MOI was 4.0. Neither parasite density nor age was related to MOI. The study concluded that extensive polymorphism exist in the *P. falciparum* population of southeast Gabon.

The study of Joshi (2003) dealt with the genetic diversity existing among the field isolates of *P. falciparum* and *P. vivax* in India. Isoenzymes: glucose phosphate isomerase (GPI), glutamate dehydrogenase (GDH), adenosine deaminase (ADA) and lactate dehydrogenase (LDH) loci in both *P. falciparum* and *P. vivax* and molecular markers: MSP-1, MSP-2 and GLURP in *P. falciparum* isolates and Circumsporozoite protein (CSP), MSP-3α and GAM-1 (transmission blocking candidate antigen) genes in *P. vivax* isolates were used to analyze field isolates by nested PCR based method. In study populations a high proportion of isolates (up to 60%) comprised multiclonal parasite type. A single random mating population of *P. falciparum* and *P. vivax* were reported in different geographical areas and in different years in that study.

Hoffman *et al.* (2003) studied the genetic relatedness of *P. falciparum* isolates and the origin of allelic diversity at the MSP-1 locus in Brazil and Vietnam. MSP-1 alleles were characterized by combining PCR typing with allele-specific primers and partial DNA sequencing. The following single-copy microsatellite markers were typed: *Polyα, TA42* (only for Brazilian samples), *TA81, TA1, TA87, TA109* (only for Brazilian samples), *2490, ARAII, PfG377, PfPK2, and TA60*.

Hay *et al.* (2004) published a review on the past, present, and future global distribution and population at risk of malaria. They reviewed the spatial distribution of malaria and human populations at risk through time. They also considered the global malaria control programs from 1900 to 2002 and the consequences of changing global population at risk of malaria in their review article.

Pearce *et al.* (2004) published a work entitled *P. falciparum* Merozoite Surface Protein 6 is a dimorphic antigen. They described the sequence of MSP-6 with their processing and importance in RBC invasion.

Terrientes *et al.* (2005) investigated restricted genetic diversity of *P. falciparum* MSP-1 in isolates from Buenaventura, Colombia. Four MSP-1 gene types were detected corresponding to prototype and recombinant K1 and MAD20 block 4 sequences by

nested PCR based methodology. In contrast to variability within block 4, blocks 2, 6, and 16–17 corresponded exclusively to the MAD20 allelic type. Most (80%) blood samples contained multiple MSP-1 gene types. The presence of four MSP-1 variants within block 4 against a MAD20 background indicates that *P. falciparum* populations in Buenaventura were derived from parasites expressing K1 and MAD20 alleles, some of which underwent two recombination events within or flanking block 4.

Farooq *et al.* (2006) carried out a study on polymorphism on the MSP-1 gene of *P. falciparum* in Indian isolates with cerebral and non-cerebral malaria. They found 69 isolates or clones of five allelic families on the basis of the molecular weight of the PCR products. They further subdivided the isolates of three allelic types by PCR-RFLP into two sub-allelic types each and thus they identified eight different genetic types. They also observed that, comparatively more virulent isolates prevalent in an area may cause cerebral malaria which can be identified by molecular techniques like PCR-RFLP.

Falk *et al.* (2006) compared the efficacy and accuracy of PCR-RFLP and GeneScan based nested PCR genotyping methods in analyzing infection dynamics of *P. falciparum*. The work revealed that the two methods used in the genotyping of the genes of *P. falciparum* gave broadly similar estimates of parasite dynamics. But GeneScan is more precise and can achieve a higher throughput. The analysis of parasite dynamics indicated an average duration of infection of 210 days by GeneScan versus 152 days by PCR-RFLP in the study population in Kassena-Nankana, Northern Ghana. This reflects the good performance of the GeneScan-based genotyping for studies of parasite infection dynamics.

Recombination-based diversity of MSP-1 in Guadalcanal, the Solomon Islands, where malaria transmission is high was monitored by Sakihama *et al.* (2006) using nested PCR based methodology. They identified 5’ recombinant types, 3’ sequence types, and MSP-1 haplotypes (unique associations of 5’ recombinant types and 3’ sequence types), and compared them with those from areas of low transmission in Thailand and Vanuatu. The mean number of 5’ recombinant types per person (multiplicity) was lower in Guadalcanal than in Thailand. Guadalcanal populations had 6–8 MSP-1 haplotypes; the numbers are comparable to Vanuatu but much lower than in Thailand. There were marked geographic differences in distribution of MSP-1 haplotypes.

Linkage disequilibrium in msp1 was stronger in Guadalcanal than in Thailand, suggesting limited recombination events in the Solomon Islands.

The work entitled '*P. falciparum* Merozoite Surface Protein 3 is a target of allele-specific immunity and alleles are maintained by natural selection' was published by Polley *et al.* (2007). They described the structure of MSP-3 with their importance in vaccine development.

Heidari *et al.* (2007) studied the genetic diversity in msp1 and msp2 genes of *P. falciparum* in a major endemic region of Iran using nested PCR amplification. Among the 67 microscopically positive study subjects, 9 alleles of msp1 and 11 alleles of msp2 were identified. The results showed that amplified product from these surface antigen genes varied in size, for K1 allele of msp1 160-220bp, for MAD20 150-210bp and for RO33 allele 160bp and for FC27 allele of msp2 200-400bp and for 3D7 allele 400-600bp was observed. Besides, regarding this pattern, 23 multiple infections with at least 2 alleles were observed in this study.

A preliminary study of genetic diversity of MSP-1 types in *P. falciparum* in southern province of Sistan Baluchistan of Iran was done by Zahra *et al.* in 2007. They genotyped the samples by nested PCR. Among the 30 febrile patients 98 clones of 19 distinct alleles were observed.

Recombination driven allelic diversity and temporal variation of *P. falciparum* MSP-1 was studied in Tanzania by Tanabe *et al.* (2007) using nested PCR and directly sequenced by ABI sequencer. The sequence was verified by re-sequencing the PCR products independently amplified from the same DNA. They identified 5' recombinant types, 3' sequence types, and MSP-1 haplotypes (unique associations of 5' recombinant types and 3' sequence types) to measure the extent and temporal variation of msp1 allelic diversity. The results show that MSP-1 haplotype diversity is higher in Tanzania as compared with areas with lower transmission rates.

Allelic diversity in the MSP-1 gene of *P. falciparum* on Palawan Island, the Philippines was investigated by Sakihama *et al.* (2007). They identified the 5′ recombinant types, 3′ sequence types and MSP-1 haplotypes (unique combinations of 5′ recombinant type and 3′ sequence type), and compared them with those of *P. falciparum* from the Solomon Islands, where malaria transmission is high. The mean

number of 5′ recombinant types per patient in Palawan was 1.44, which is comparable to the number for the Solomon Islands (1.41). The Palawan parasite population had 15 MSP-1 haplotypes, whereas the Solomon Islands population had only 8 haplotypes. The Palawan population showed strong linkage disequilibrium between polymorphic blocks/sites within MSP-1, which is comparable to the results for the Solomon Islands.

In 2007 Joshi *et al.* investigated the genetic structure in MSP-1 and MSP-2 gene of *P. falciparum* field isolates in eastern and north-eastern India using allele specific nested PCR assays and sequence analysis. Field isolates were collected from five sites distributed in three states namely, Assam, West Bengal and Orissa. *P. falciparum* isolates of the study sites were highly diverse in respect of length as well as sequence motifs with prevalence of all the reported allelic families of MSP-1 and MSP-2. Prevalence of identical allelic composition as well as high level of sequence identity of alleles suggest a considerable amount of gene flow between the *P. falciparum* populations of different states.

Cano *et al.* (2007) investigated the transmission pattern of malaria and genotypic variability in MSP-1 and MSP-2 of *P. falciparum* on the Island of Annobon in Equatorial Guinea by semi-nested multiplex PCR. A total of 28 populations of the three allelic families of the MSP-1 gene were identified and 39 of the MSP-2 gene. The variability of circulating allelic populations is significantly higher in the rainy than in the dry season, although the multiplicity of infections is similar in both, 2.2 and 1.9 respectively.

Baruah *et al.* (2009) studied the temporal and spatial variation in MSP-1 clonal composition of *P. falciparum* in districts (Guabari, Kondoli and Dimakusi) of Assam, Northeast of India. The study was conducted during the malaria outbreak of 2006 and of 2007. MSP1 diversity expressed as mean number of distinct alleles per isolate was 0.68 at Dimakusi and was much higher ($p = 0.007$) than seen at Guabari (0.336) and Kondoli (0.45) as was multiplicity of infection at 4.12, indicating the highest diversity at Dimakusi. Size polymorphism of the allelic families at Guabari was distinctly different from Kondoli but shared similarity with Dimakusi. Infections in high transmission summer season tended to be more complex with higher number of alleles. The frequency of alleles of RO33 and MAD20 allelic families at Guabari was

found to be different between the two transmission periods. A 380 base pair allele of RO33 was over represented in high transmission summer season and seen frequently in isolates with high parasitaemia. At Kondoli allele distribution of only MAD20 was found to be different in each study year. The study demonstrated that there exists spatial and temporal variation in allelic composition of *P. falciparum*.

Soulama *et al.* (2009) worked on the genetic diversity of *Plasmodium falciparum* in symptomatic malaria of children in an urban and rural setting in Burkina Faso using two polymorphic markers, namely, msp1 and msp2; of 75 and 89 from each setting, respectively, by nested PCR amplification. In the study, 70 individual msp alleles were identified in the study children in the two sites: 16 different alleles for FC27 (150-900bp); 13 for 3D7 (150-800bp0; 13 K1 types allele (100-500bp); 11 alleles for RO33 (120-550bp) and 17 MAD20 types allele (100-600bp). The study demonstrated that Urban-rural area differences in some allelic families (MAD20, FC27, 3D7), suggesting a probable impact of urbanization on genetic variability of *P. falciparum*.

Schoepflin *et al.* (2009) compared the allelic frequency distribution of MSP-1 and MSP-2 gene of *P. falciparum* in different endemic settings of Papua New Guinea and Tanzania by a high resolution genotyping method (Capillary electrophoresis). They observed 24 and 35 distinct allels of MSP-1 and MSP-2 gene respectively, with mean Multiplicity of Infection (MOI) of 1.99 and 1.84, while number of distinct alleles for MSP-1 and MSP-2 gene of Tanzania was 29 and 76, respectively, with mean MOI of 3.04 and 3.72.

Kiwanuka *et al.* (2009) published a review work on the genetic diversity in *P. falciparum* MSP-1 and MSP-2 coding genes and its implications in malaria epidemiology. This review work provides current and comprehensive information on the diversity in the gene that encodes the MSP-1 and MSP-2 of *P. falciparum* and its implications on the epidemiology of malaria, immunity and development of control measures, and point out some research themes that need to be explored further by utilizing molecular techniques currently at our disposal.

Bonizzoni *et al.* (2009) investigated prevalence, genetic variability and population genetic structure of *P. falciparum* in asymptomatic children from one highland site and three surrounding malaria endemic lowland sites in Western Kenya, using

multilocus microsatellite genotyping. Ten microsatellite markers (TA1, TA42, TA81, TA87, TA109, ARA2, 2490, Polyα, PfPK2 and Pfg377) were used in this study. They also analyzed the frequencies of mutations at the genes conferring resistance to chloroquine and sulfadoxine–pyrimethamine.

Genetic diversity among *P. falciparum* field isolates in Pakistan measured with PCR genotyping of the merozoite surface protein 1 and 2 was done by Ghanchi *et al.* (2010). A total of 238/244 (98%) patients had a positive PCR outcome in at least one genetic marker; the remaining six were excluded from analysis. A majority of patients had monoclonal infections. Only 56/231 (24%) and 51/236 (22%) carried multiple *P. falciparum* genotypes in MSP-1 and MSP-2, respectively. The estimated total number of genotypes was 25 MSP-1 (12 KI; 8 MAD20; 5 RO33) and 33MSP-2 (14 FC27; 19 3D7/IC).

Kang *et al.* (2010) investigated the genetic polymorphism of MSP-1 and MSP-2 in *P. falciparum* field samples from Myanmar by allele-specific nested-PCR. They also conducted sequence analysis to identify allelic diversity in the parasite population. Diverse allelic polymorphism of MSP-1 and MSP-2 was identified in *P. falciparum* isolates from Myanmar and most of the infections were determined to be mixed infections. Sequence analysis of MSP-1 block 2 revealed that 14 different alleles for MSP-1 (5 for K1 type and 9 for MAD20 type) were identified and for MSP-2 block 3, a total of 22 alleles (7 for FC27 type and 15 for 3D7 type) were identified.

CHAPTER 3

OBSERVATION ON EPIDEMIOLOGY OF MALARIA IN BANGLADESH

Materials and Methods

Study areas:

The present study was conducted in 13 malaria endemic districts of Bangladesh, namely: Sherpur, Mymesingh, Netrokon, Kurigram, Sylhet, Hobigonj, Sunamgonj, Moulvibazar, Chittagong, Khagrachari, Rangamati, Bandarba and Cox's Bazar. Malaria is reported only from these 13 districts of Bangladesh within recent years (Figure 3.1).

Study Populations:

Malaria case data during January 2010- December 2013, from 13 malaria endemic districts were collected with the permission of corresponding District Civil Surgeon's Office on monthly basis. Available data included the number of persons diagnosed as malaria patients by either microscopy or by Rapid Diagnostic Test (RDT), age group, sex, type of infection etc. All collected data were checked for the reliability and consistency with the National Malaria Control Programme (NMCP).

Data Management:

The collected data were computed and maintained by using Microsoft Excel software in a regular basis. After completion of data entry all files were converted to specialized statistical software such as Statistical Package for the Social Sciences (SPSS) verified and analyzed.

Data Analysis:

Annual incidence of malaria for 2010-2013 (4 years) was calculated at first at the national level and then according to district level. Later data were analyzed for other parameters such as: gender, age group (<1 year, 1-4 years, 5-14 years and 15 years plus) as per WHO classification, types of parasites involved etc. After analyzing by SPSS, raw tables were formatted in Microsoft Excel to convert final tables and graphs in presentable ways.

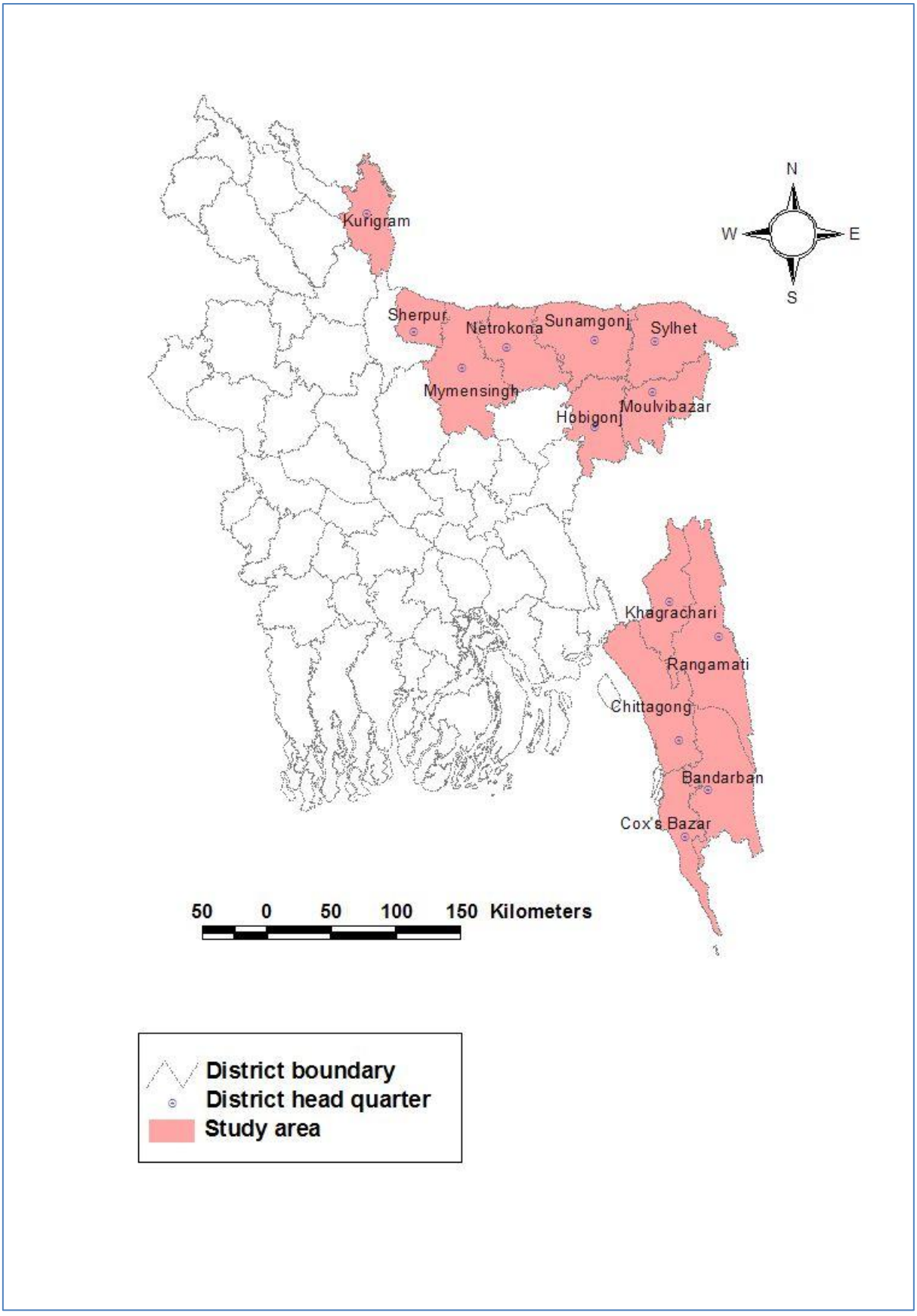


Figure 3.1: Selected areas for malaria epidemiological study shown in a map of Bangladesh.

Results and Observations

Annual Malaria Incidences:

During the study period (2010-13), a total of 1,64,055 malaria cases were reported from 13 endemic districts of Bangladesh. Highest number of cases 55,873 was reported in the year 2010 followed by 51,773 in 2011, 29,518 in 2012 and 2013 (26,891) respectively (Figure: 3.2).

Prevalence of malaria according to sex and age groups:

During the study period, a total of 96,189 male and 67,866 female were identified as malaria patients. The number of male vs. female infection was 31,669 and 24,024 in 2010; 30,531 and 21,242 in 2011; 17,621 and 11,897 in 2012 and 16,368 and 10,523 in 2013 respectively (Figure: 3.3). Male are more prone to malaria infection than female. The overall mean female: male ratio of annual malaria incidence was1:1.42. However this rate was 1:1.31 in 2010; 1: 1.44 in 2011; 1:1.48 in 2012 and 1:1.56 in 2013 respectively.

According to age group, highest malaria incidence was always observed in working age group 15+ years. A total of 100,413 (%) malaria cases were reported from this age category. This number is followed by 44,823 (%) in 5-14 years of age group, 17,628 (%) in 1-4 years of age group and 1,191 in less than one year age group respectively (Figure: 3.4).

Types of malaria infection:

Malaria caused by *Plasmodium falciparum* was found dominant in Bangladesh. A total of 154,562 (94.2%) *P. falciparum* infected cases were detected during the study period (Figure 3.5). Only 5.5% cases were reported to be caused by *P. vivax*. Remaining infection was caused by *P. falciparum* and *P. vivax* mixed infection (Figure 3.6)

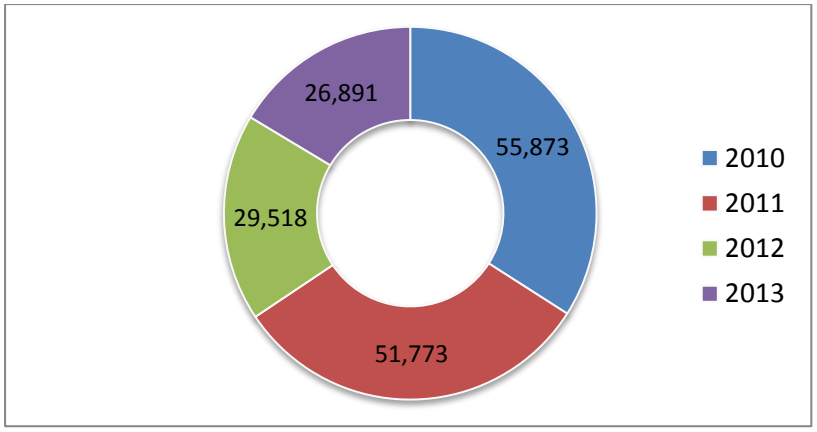


Figure 3.2: Incidence of annual malaria cases in Bangladesh during 2010-13.

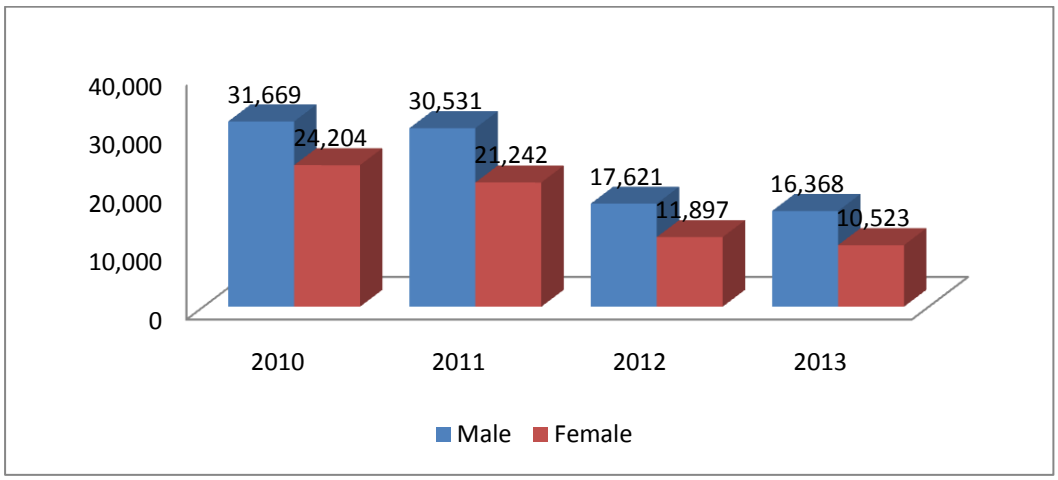


Figure 3.3: Incidence of annual malaria cases in male and female in Bangladesh during 2010-13.

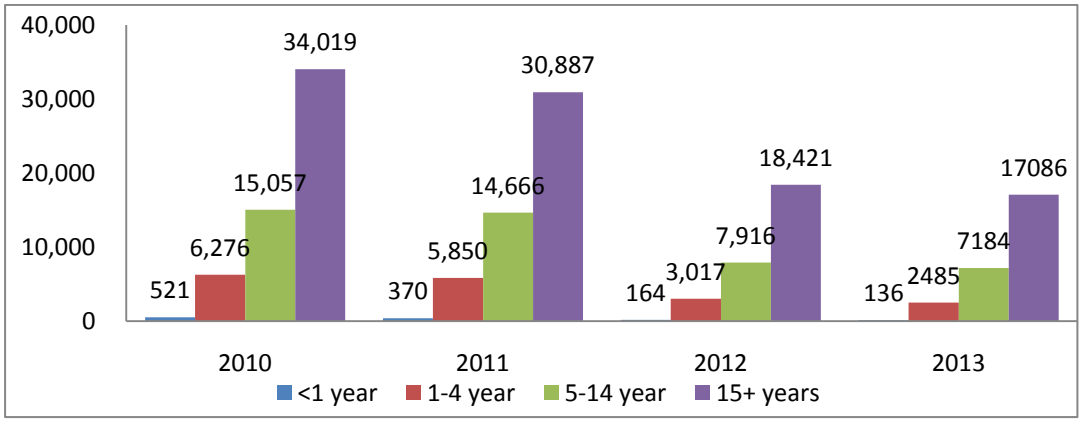


Figure 3.4: Prevalence of malaria cases according to age groups during 2010-2013.

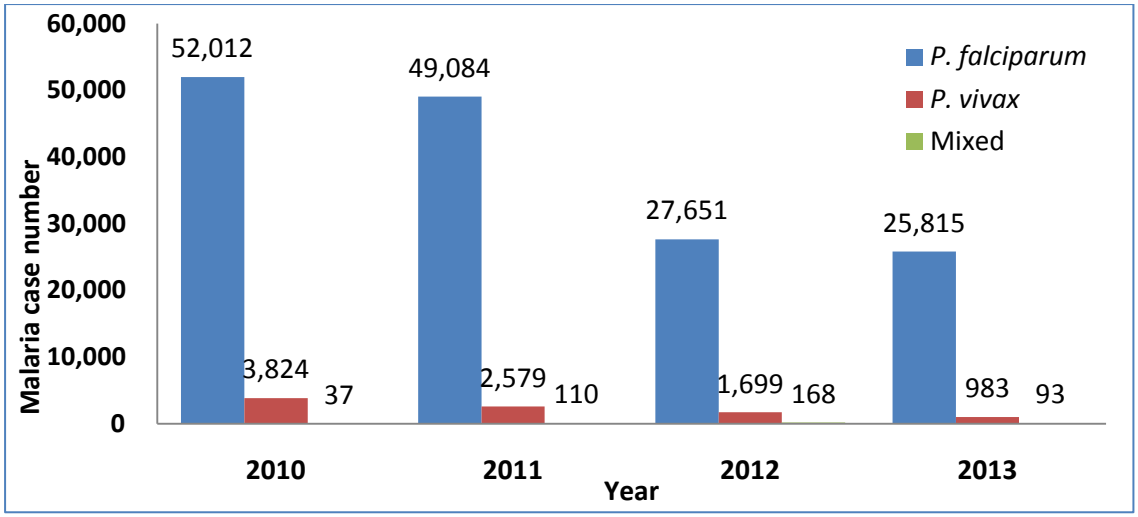


Figure 3.5: Prevalence of malaria infections by different *Plasmodium* spp. during 2010-13.

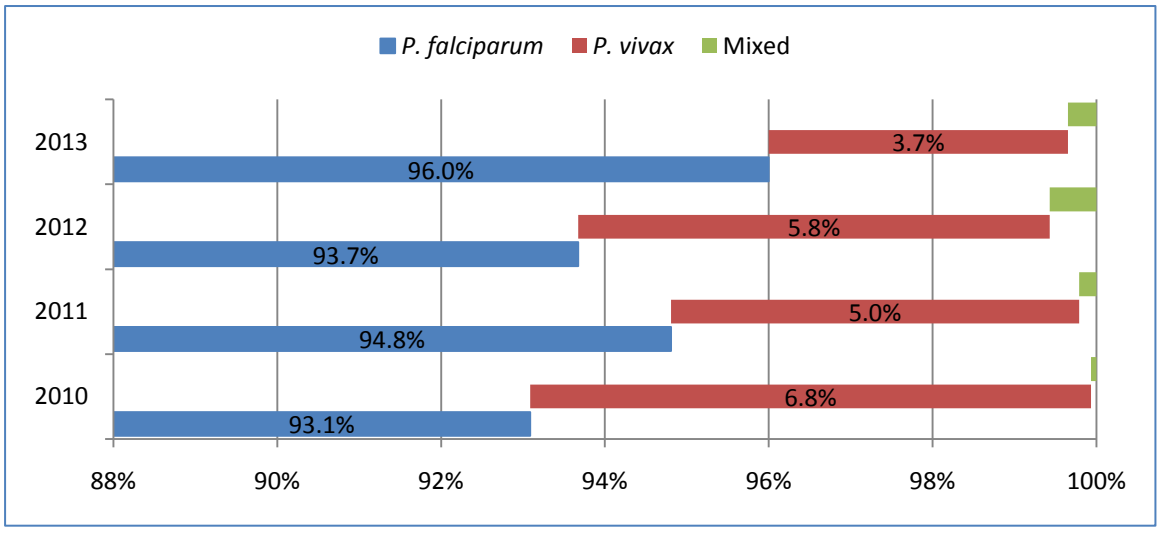


Figure 3.6: Percentage of malaria patients infected with different species during 2010-13 in Bangladesh.

Malaria in Pregnancy:

Malaria infection during pregnancy is a significant public health problem with substantial risks for the pregnant woman, her fetus, and the newborn child. Malaria-associated maternal illness and low birth weight is mostly the result of *P. falciparum* infection. During the study period a total of 497 malaria cases were identified among pregnant women in endemic areas of Bangladesh. Highest number of pregnant women (153) was affected in the year 2011 and lowest (106) in 2013. *P. falciparum* remains the main causing agent for malaria during pregnancy. The highest number of *P. falciparum* infection among pregnant women was 140 in 2011 and lowest 106 in 2013. The prevalence of *P. falciparum* infecting women ranges between 88.6%-91%. The highest number of *P. vivax* infection among pregnant women was 14 in 2010 and lowest 0 in 2013. Overall contribution of *P. vivax* infected malaria among pregnant women ranges from 9.3%-12.8% during this period (Figure: 3.7).

Death due to malaria:

Malaria is a leading cause of morbidity and mortality in many developing countries. However, in our country at present do not have any data related to morbidity due to malaria but mortality data are recorded. Among human malaria parasites *P. faliparum* is most likely to cause severe malaria and death. A total of 99 patients died during this period (2010-13). Quiet opposite of the malaria incidence, female (51) showed slightly higher death than male (48). Highest number of death was 37 in 2010 followed by 36 in 2011 (Table 3.2). Lowest number of death due to malaria was 11 in 2012 followed by 15 in 2013. Thus, case fatality rate during 2010-13 was 7.1, 7.3, 4.0 and 5.8 respectively per 10,000 *P. falciparum* malaria infected person that also include mixed infection (Table 3.2 and Figure: 3.9). However, for female this rate became 8.0; 10.8; 3.5 and 6.8 respectively from 2010-13. Male only had high mortality rate over female in 2012. During remaining year (2010- 11 and 2013), female had higher fatality rate due to severe malaria (Figure: 3.9).

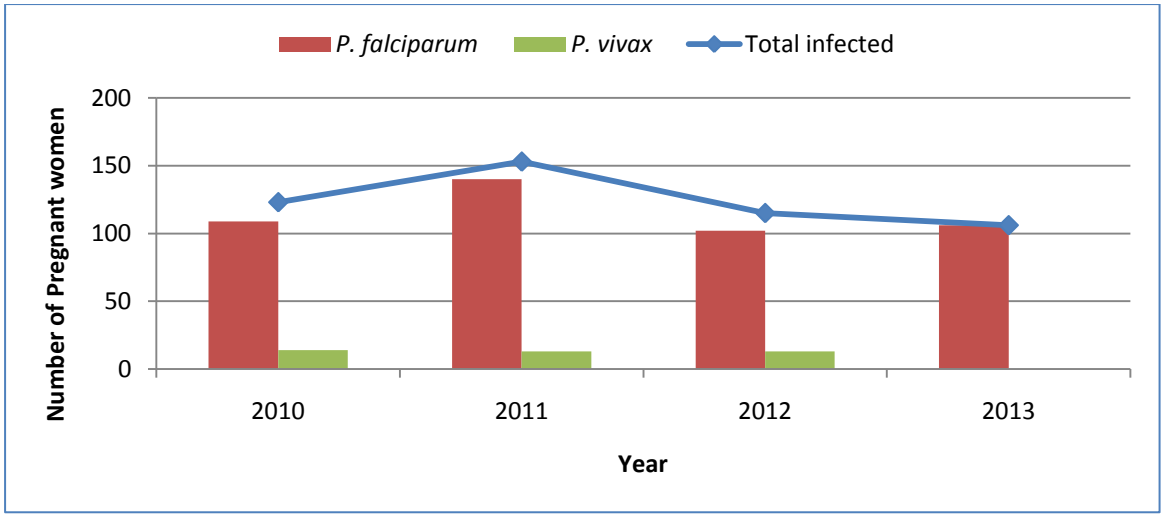


Figure 3.7: Malaria incidence in pregnancy during 2010-13 in Bangladesh.

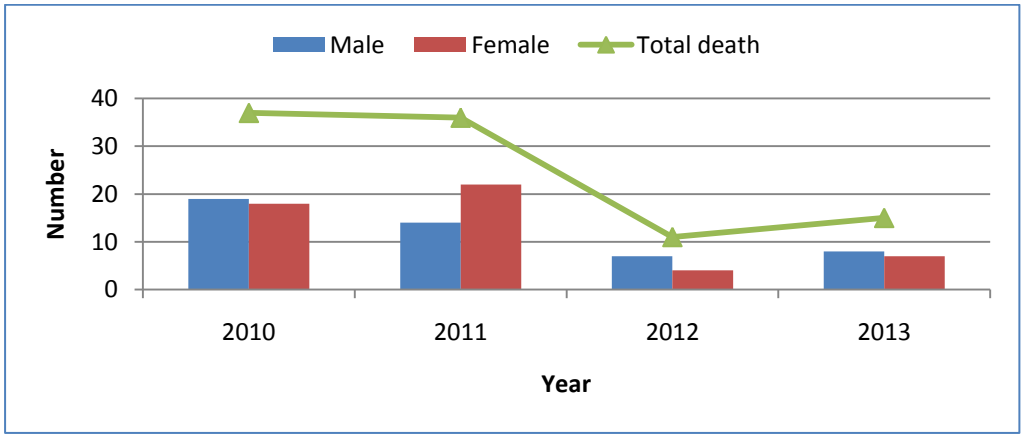


Figure 3.8: Death due to malaria in different sexes during 2010-13 in Bangladesh.

Table 3.1: Death associated with *P. falciparum* infection in different sexes during 2010-13.

Year	Male			Female		
	Case No.	Death No.	Death rate	Case No.	Death No.	Death rate
2010	29,476	19	6.4	22,573	18	8.0
2011	28,876	14	4.8	20,318	22	10.8
2012	16,539	7	4.2	11,280	4	3.5
2013	15,680	8	5.1	10,228	7	6.8
Total	90,571	48	5.3	64,399	51	7.9

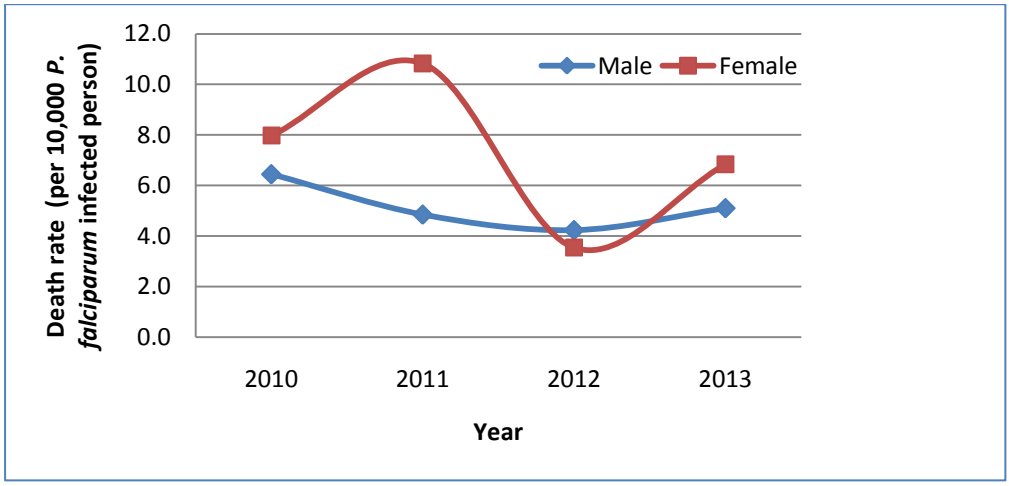


Figure 3.9: Death rate per 10,000 by *P. falciparum* infection in male and female during 2010-13.

Malaria in different districts:

Bandarban district recorded the highest malaria burden in Bangladesh. The numbers of cases in all four years were 17,259 in 2010, 16,097, 8,461 and 9,459 in 2011, 2012, and 2013 respectively. Rangamati district remains second position throughout this period with 13,949; 13,669; 7,981 and 7,976 respectively during 2010-13. Khagrachhari district remain the third position with 12,348; 12,952; 5,997 and 4,096 cases respectively during 2010-13 (Table 3.2 and Figure 3.10).

Table 3.2: Malaria incidence in different districts during 2010-13.

Sl	**District**	**2010**	**2011**	**2012**	**2013**	**Total**
1	Sherpur	72	68	73	43	256
2	Mymensingh	418	244	168	74	904
3	Netrokona	375	270	285	199	1129
4	Kurigram	337	168	101	64	670
5	Sylhet	882	444	436	360	2122
6	Hobigonj	120	65	72	34	291
7	Sunamgonj	1134	461	540	488	2623
8	Moulvibazar	944	448	421	198	2011
9	Chittagong	1,621	1,415	1,095	648	4,779
10	Khagrachari	12,348	12,952	5,997	4,096	35,393
11	Rangamati	13,949	13,669	7,981	7,976	43,575
12	Bandarban	17,259	16,097	8,461	9,459	51,276
13	Cox's Bazar	6,414	5,472	3,888	3,252	19,026
	Total	**55,873**	**51,773**	**29,518**	**26,891**	**164,055**

Apart from these three districts of Chittagong Hill Tracts (CHT) significant number of malaria cases were reported in Cox's Bazar district with 6,414; 5,472; 3,888 and 3,252 cases respectively followed by Chittagong with 1,624; 1,415; 1,095 and 648 cases respectively from 2010-13 (Table 3.2 and Figure 3.10).

In Sylhet zone highest number of cases were reported from Sunamgonj district with 1,134; 461; 540 and 488 cases respectively from 2010-13, followed by Sylhet district (882; 444;436 and 360 respectively (Table 3.2 and Figure 3.10).

In northern zone, the highest numbers of cases were reported from Netrokona district with 375; 270; 285 and 199 cases respectively followed by Mymensing district with 418; 244; 168 and 74 malaria cases respectively during 2010-13 (Table 3.2 and Figure 3.10).

Chittagong Hill Tracts districts (Bandarban, Rangamati and Khagrachari) accounted for almost 80% of total malaria cases of the country. Precisely Chittagong hill tracts contributed 78% of country's total malaria cases in 2010; 82.5% in 2011; 76% in 2012 and 80.1% in 2013 respectively (Figure 3.11). Bandarban district of Chittagong hill tracts region contributed on average 31.3% cases reported in between 2010-13. This contribution remains 30.9%, 31.1%, 28.7% and 35.2% respectively during 2010-13 (Figure 3.12 to 3.15).

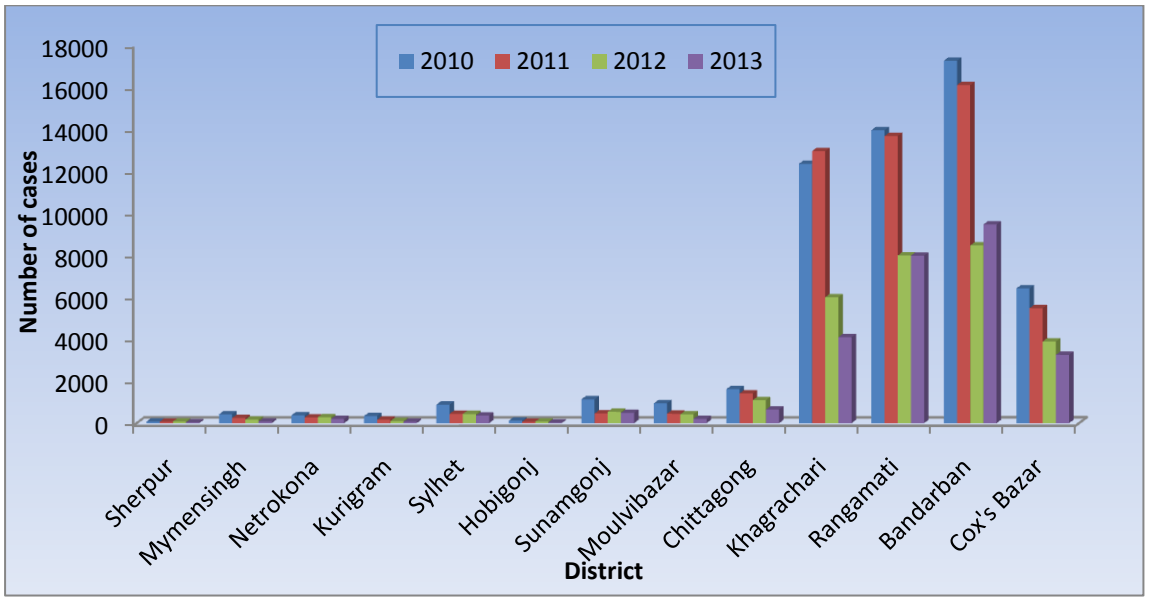


Figure 3.10: Malaria incidence during 2010-13 in different districts of Bangladesh.

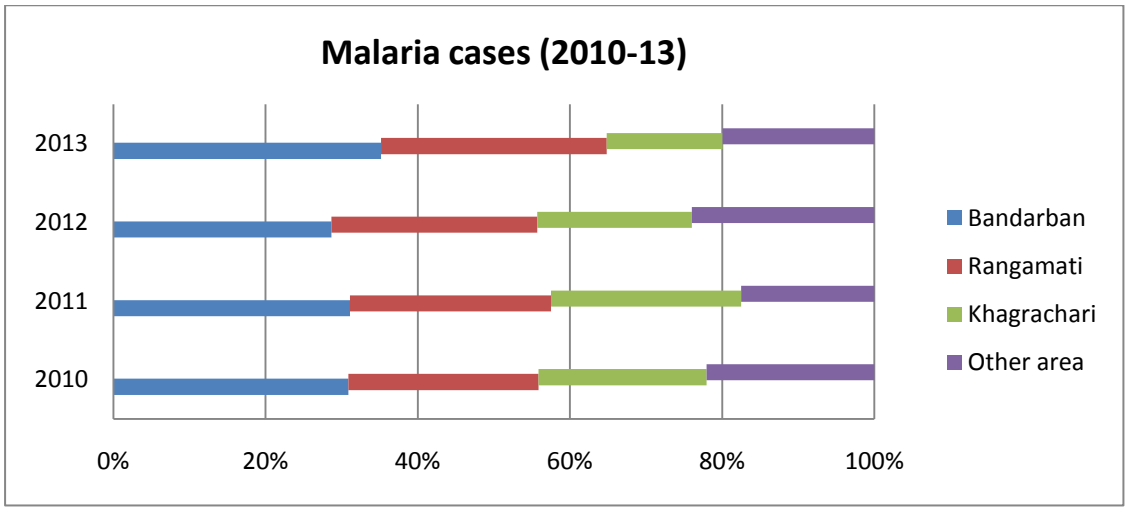


Figure 3.11: Contribution of Chittagong hill tracts districts in annual malaria incidences in Bangladesh during 2010-13.

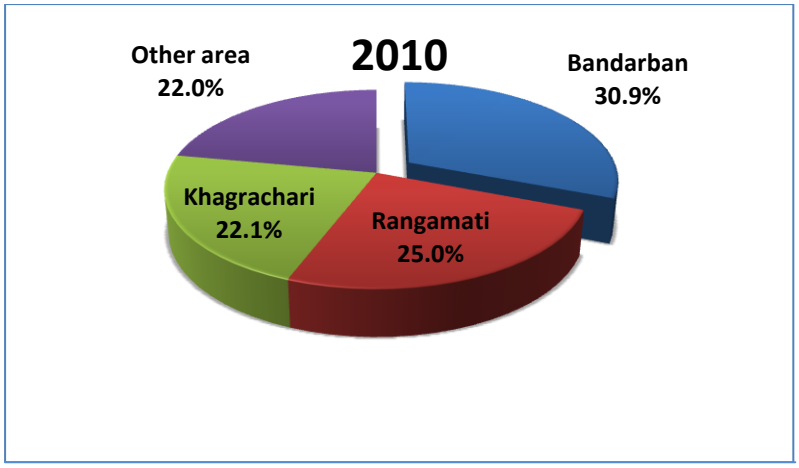


Figure 3.12: Highest malaria incidence showed by Bandarban in national malaria case burden in 2010.

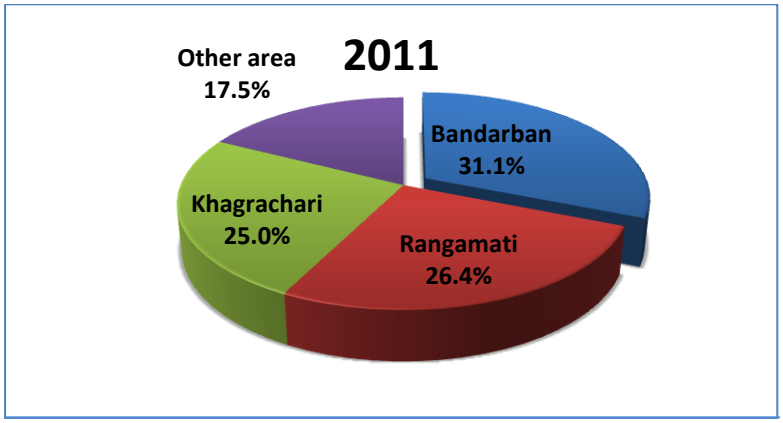


Figure 3.13: Highest malaria incidence showed by Bandarban in national malaria case burden in 2011.

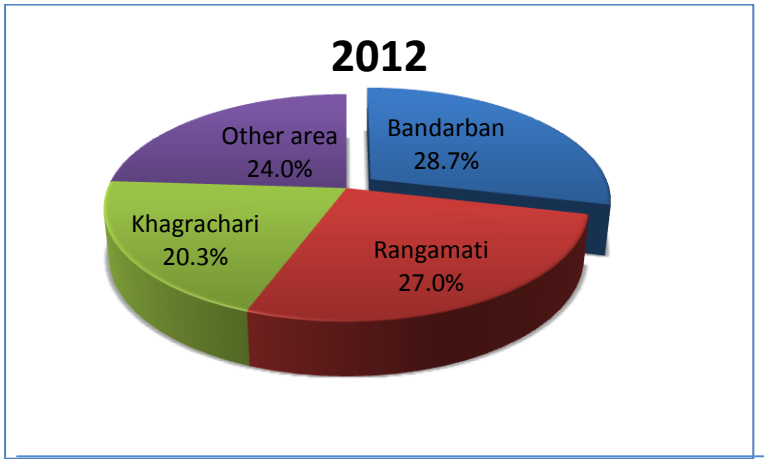


Figure 3.14: Highest malaria incidence showed by Bandarban in national malaria case burden in 2012.

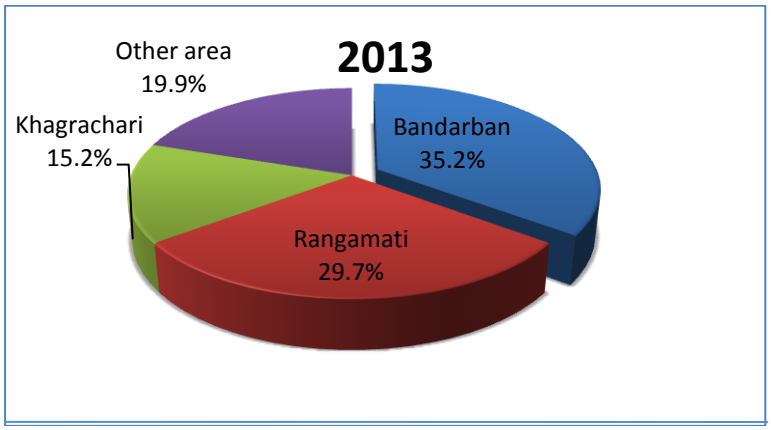


Figure 3.15: Highest malaria incidence showed by Bandarban in national malaria case burden in 2013.

Prevalence of *Plasmodium species* in different districts:

Throughout the study period, Bandarban remains as the highest *P. falciparum* burdened district in Bangladesh. A total of 49,389 (31.9%) *P. falciparum* infected malaria cases were identified in Bandarban during the period 2010-13. Bandarban also recorded highest *P. falciparum* infected malaria cases in all four years (2010-13). The second most infected district for *P. falciparum* infection was Rangamati (41,396; 26.8%) followed by Khagrachari (34,469; 22.2%). Outside of the Chittagong hill tracts, Cox's Bazar represented the highest number of *P. falciparum* cases (17,398; 11.3%) during the study period (Table 3.3 and Figure 3.16).

Throughout the study period, Rangamati district remains as the highest *P. vivax* burdened district in Bangladesh. Out of 9,043 *P. vivax* infected cases in Bangladesh, 2,092 cases were identified in Rangamati district during 2010-13. . This number accounted for 23.1% total *P. vivax* infection in the country. The second most infected district for *P. vivax* infection was Bandarban (1,671; 18.5%) followed by Cox's Bazar (1,621; 17.9%). Rangamati recorded highest *P. vivax* infected malaria cases in 2010 and 2011 but Cox's Bazar was observed to be highest in 2012 and 2013 (Table 3.4 and Figure 3.17).

According to *Plasmodium* species, all endemic districts except for Kurigram showed higher number of *P. falciparum* infection than *P. vivax* infection. However,

Khagrachari recorded the highest percentage (97.4%) of *P. falciparum* infection among total case reported during 2010-12. On the other hand Kurigram reported the highest percentage (72.6%) of *P. vivax* infection among total case report during 2010-13(Figure 3.18). Among the total case reported during 2010-13, no district was found to have equal representation of these two malaria parasite species except for Sherpur which has 2: 1 ratio represented by 67.8% of *P. falciparum* infection over 32.2% *P. vivax* infection among total case reported during 2010-13 (Figure 3.18).

Table 3.3: Incidence of *P. falciparum* in different districts during (2010-13).

Sl	**District**	**2010**	**2011**	**2012**	**2013**	**Total**
1	Sherpur	50	47	45	31	173
2	Mymensingh	400	236	167	70	873
3	Netrokona	350	236	266	189	1041
4	Kurigram	83	50	38	12	183
5	Sylhet	619	352	334	332	1637
6	Hobigonj	111	62	60	33	266
7	Sunamgonj	753	420	498	471	2142
8	Moulvibazar	685	325	283	158	1451
9	Chittagong	1333	1258	989	601	4181
10	Khagrachari	12010	12674	5773	4012	34469
11	Rangamati	12884	12959	7681	7872	41396
12	Bandarban	16732	15495	8035	9127	49389
13	Cox's Bazar	6002	4972	3517	2907	17398
	Total	**52012**	**49086**	**27686**	**25815**	**154599**

Table 3.4: Incidence of *P. vivax* in different districts during 2010-13.

Sl	District	2010	2011	2012	2013	Total
1	Sherpur	22	21	27	12	82
2	Mymensingh	18	8	1	2	29
3	Netrokona	25	34	19	7	85
4	Kurigram	253	119	63	52	487
5	Sylhet	262	92	72	28	454
6	Hobigonj	9	3	12	1	25
7	Sunamgonj	380	41	41	11	473
8	Moulvibazar	259	123	137	38	557
9	Chittagong	288	154	99	45	586
10	Khagrachari	331	273	212	65	881
11	Rangamati	1047	692	259	94	2092
12	Bandarban	521	520	345	285	1671
13	Cox's Bazar	409	499	370	343	1621
	Total	3824	2579	1657	983	9043

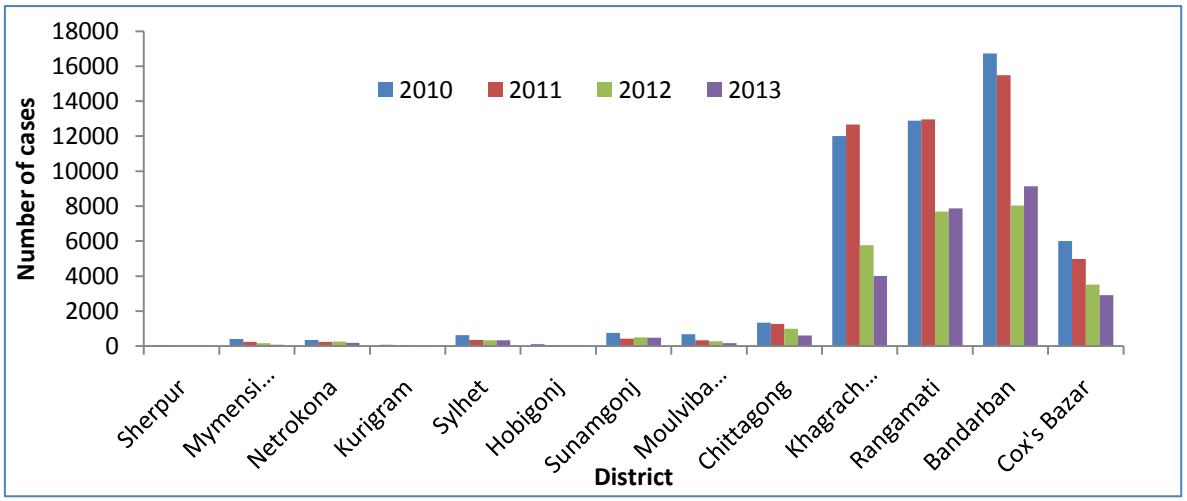


Figure 3.16: Prevalence of *P. falciparum* in different districts.

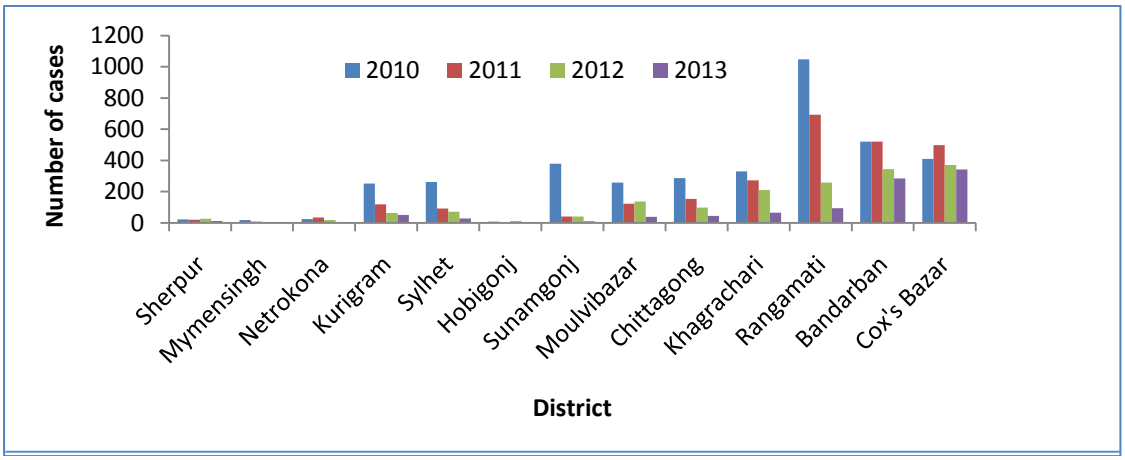


Figure 3.17: Prevalence of *P. vivax* in different districts.

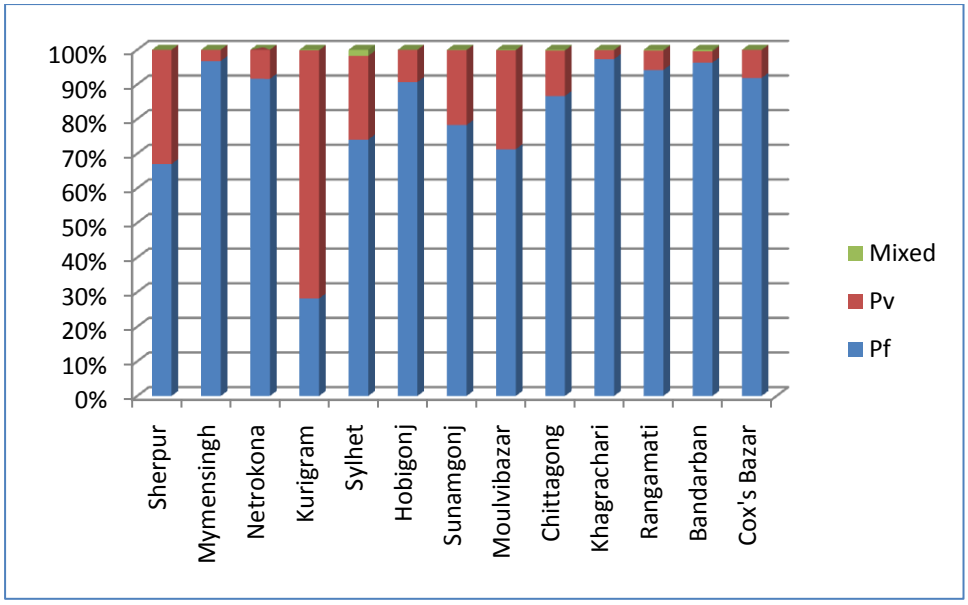


Figure 3.18: contribution of malaria parasite species in different districts.

Table 3.5: Distribution of malaria parasite species among district level.

District	Total malaria cases	Pf	Pf (%)	Pv	Pv (%)
Sherpur	255	173	67.8%	82	32.2%
Mymensingh	904	873	96.6%	29	3.2%
Netrokona	1,129	1,041	92.2%	85	7.5%
Kurigram	671	183	27.3%	487	72.6%
Sylhet	2,122	1,637	77.1%	454	21.4%
Hobigonj	291	266	91.4%	25	8.6%
Sunamgonj	2,623	2,142	81.7%	473	18.0%
Moulvibazar	2,012	1,451	72.1%	557	27.7%
Chittagong	4,779	4,181	87.5%	586	12.3%
Khagrachari	35,393	34,469	97.4%	881	2.5%
Rangamati	43,575	41,396	95.0%	2,092	4.8%
Bandarban	51,276	49,389	96.3%	1,671	3.3%
Cox's Bazar	19,026	17,398	91.4%	1,621	8.5%

(Here, Pf= *Plasmodium falciparum*, Pv= *Plasmodium vivax* and Mixed= *P. falciparum* and *P. vivax* mixed infection).

Temporal distribution of Malaria in Bangladesh

Malaria has been observed to be prevalent throughout the year in Bangladesh. However, an increasing trend or a peak of transmission was found in between May-October. More precisely, malaria incidence was found highest during June- July of a particular year. During 2010 -2011, June was the most prevalent month (7554 and 8478 respectively) while July was the most malaria prevalent month in 2012 and 2013 (4333 and 5299 respectively). Overall, in July 15.4% and June 14.3% of country's total malaria cases were recorded. The lowest monthly incidence was recorded in March (4.4%) followed by February (4.7%) during 2010-13 (Table 3.6 and Figure 3.19). An overall monthly malaria trend of malaria in recent years is presented in Figure 3.20.

Table 3.6: Monthly malaria cases recorded in Bangladesh during 2010-13.

Month	**2010**	**2011**	**2012**	**2013**	**Total**	**Average**	**Pre (%)**
Jan	3919	2737	2166	1220	10042	2511	6.1%
Feb	3249	1997	1624	857	7727	1932	4.7%
Mar	3313	2033	1272	667	7285	1821	4.4%
Apr	3697	2392	1283	752	8124	2031	5.0%
May	4264	3989	1839	1230	11322	2831	6.9%
Jun	7554	8418	3529	3993	23494	5874	14.3%
Jul	7494	8166	4333	5299	25292	6323	15.4%
Aug	6213	6423	2547	3350	18533	4633	11.3%
Sep	4163	4948	3239	2792	15142	3786	9.2%
Oct	4234	4355	2959	2431	13979	3495	8.5%
Nov	4377	3647	2562	2281	12867	3217	7.8%
Dec	3396	2668	2165	2019	10248	2562	6.2%
Total	**55873**	**51773**	**29518**	**26891**	**164055**	**41014**	**100.0%**

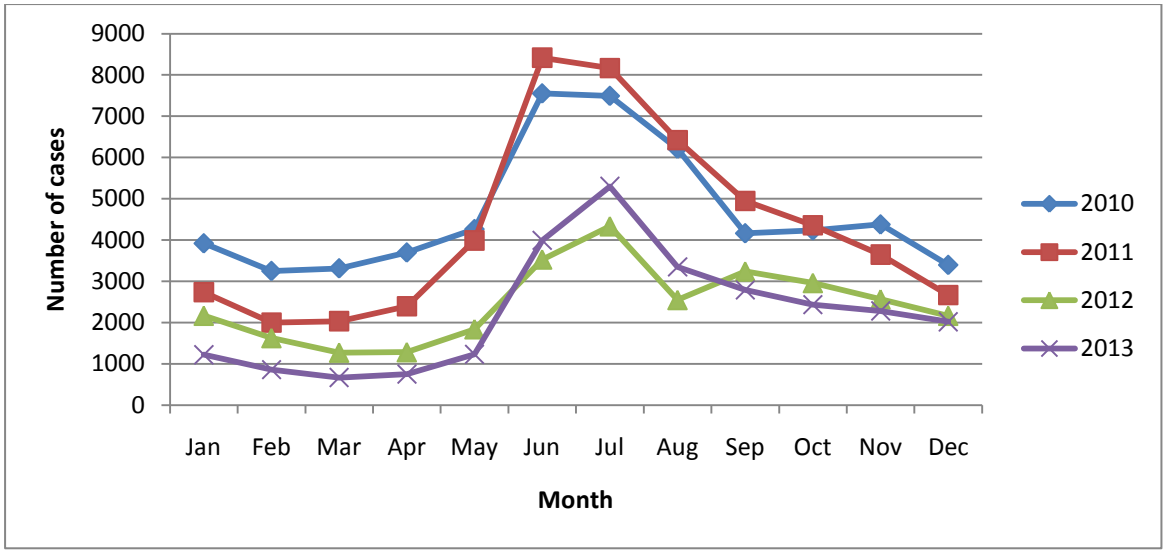


Figure 3.19: Monthly incidences of malaria cases during 2010-13.

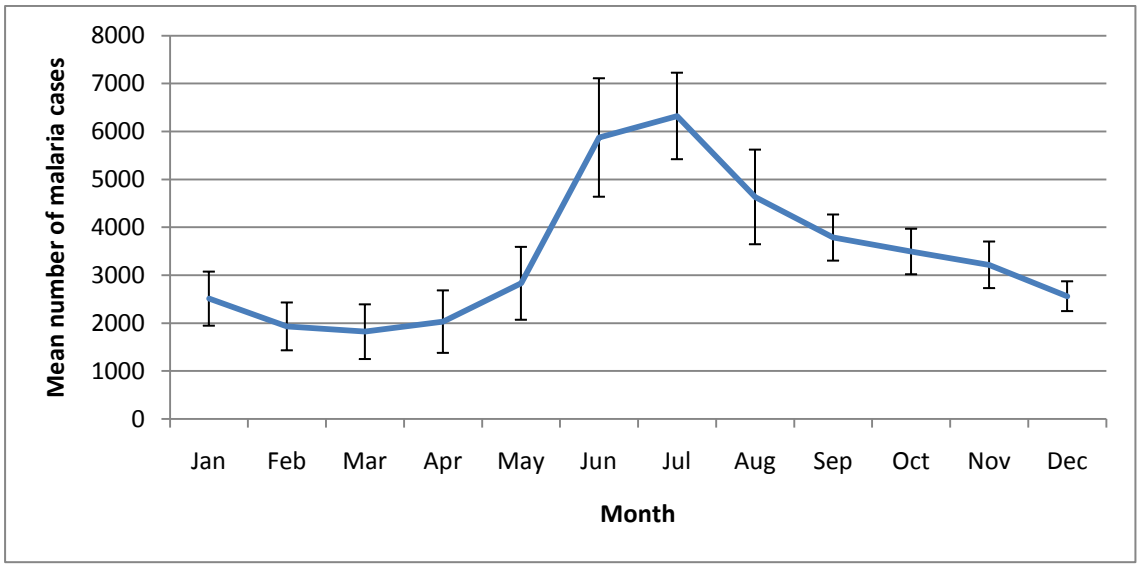


Figure 3.20: Temporal distribution trend of malaria in Bangladesh (2010-13) with standard error (SE).

Overall trend of malaria

A decreased trend for malaria transmission has been seen during 2010-13 from previous years. In Bangladesh, from 2009-2010, on average annual malaria incidence was 57,587 people per year whereas; during 2010-13 the average annual incidence was found 41,014 people per year. The average annual malaria incidence for most recent four years (2006-2009) was 60,319 people per year. Thus, in all aspect in recent years a decreasing trend for malaria incidence was evident in Bangladesh (Figure 3.21).

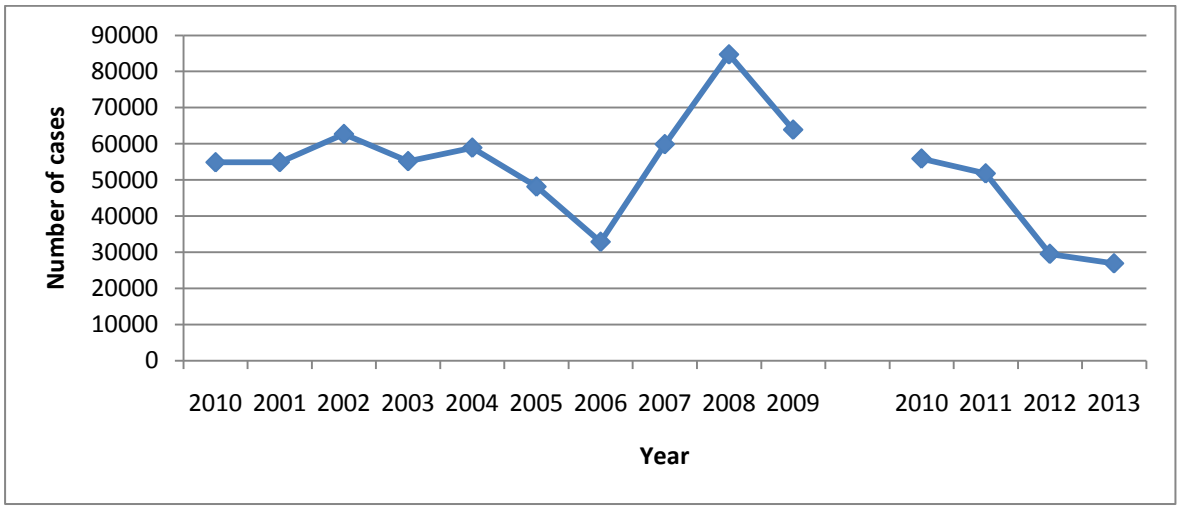


Figure 3.21: Malaria incidence in Bangladesh from 2000-2013 which clearly indicating a decreasing trend in recent years.

Discussion

Annual malaria incidences:

Before the introduction of the Malaria Eradication Program (MEP) in 1961, the disease was so widespread throughout the country (Robinson 1967). The incidence of malaria in Bangladesh was much higher in the years 1972-1977, which may be termed as the resurgence period, than in the years 1968-1971, which may be termed as the waning period (Paul 1984). However, the MEP was suspended during July 1977 and merged with Malaria and Parasitic Disease Control (M&PDC) Programme. During MEP period, DDT was used as indoor residual spray (IRS) which contributed a rapid reduction of malaria vector and thereby malaria incidence. However, due to its long residual and harmful activity to human health and environment DDT was banned in 1985 and withdrawn from public health application such malaria control activity. As a result, a resurgence of malaria occurred in 90's. According to M&PDC annual malaria incidence increased from 3.2 per 1000 population to 14.2 per 1,000 populations in 1994 and 12.8 in 1995 with some periodic epidemics. This incidence rate again came down in a stable rate in between 1996-2007. During the study period (2010-13), a total of 1,64,055 malaria cases were reported from 13 endemic districts of Bangladesh (Figure: 3.2).

In first ever national malaria prevalence survey reported 3.1% overall malaria prevalence rate among 13 malaria endemic districts. The prevalence of *P. falciparum* was 2.73% and that for *P. vivax* was 0.16%. Mixed *P. falciparum* / *P. vivax* infections had a prevalence of 0.19%. The proportion of *P. falciparum* in all parasite positive samples was 88.6%. The overall malaria prevalence in Chittagong Hill Tracts areas was found11% (Haque *et al.* 2009a).

In 2007, Bangladesh received Global Funds to Fight AIDS, Tuberculosis and Malaria to support malaria control activity. As a consequence Malaria incidence rate decreased from 6·2 cases per 1000 population in 2008, to 2·1 cases per 1000 population in 2012 (Haque *et al*. 2014).This trend also remained evident in 2013 as the incidence was found 2.0 in that year.

The number of confirmed malaria cases reported in the South-East Asia region decreased from 2.9 to 2 million between 2000 and 2012. Three countries accounted

for 96% of reported cases in 2012: India (52%), Myanmar (24%) and Indonesia (22%) (WHO 2013).

Malaria in sexes and age groups:

Available evidence suggests that given equal exposure, adult men and women are equally vulnerable to malaria infection (Reuben 1993). Historically male are prone to malaria in Bangladesh. Malaria prevalence among male individuals was 1·3 times higher than in female individuals during 2008-12 (Haque *et al.* 2014). However, during first malaria prevalence survey in Bangladesh, the prevalence in female (3.98%) was marginally higher than male (3.96%) (Haque *et al.* 2009a).

In some societies, men have a greater occupational risk of contracting malaria than women if they work in mines, fields or forests at peak biting times, or migrate to areas of high endemicity for work (Ruben *et al*. 1993). This is particularly true for Chittagong Hill Tract who jhum cultivation, rubber and tick plantation, wood logging remain main occupation among rural and tribal male. In Bandarban we found this practice to be associated with an increased risk (odds ratio of 1.5) compared to other occupation groups (Ahmed *et al*. 2013 and Galagan *et al*. 2014). Importantly, this increased risk extended to household members of jhum families among family members who do not practice jhum relative to households without one doing jhum cultivation (odds ratio = 2.3), suggesting that the jhum workers act as a reservoir and bring malaria back to their homes and community (Galagan *et al.* 2014).

Women often have to ask for their husband's permission to access treatment for themselves and/or their children (Molyneux *et al*. 2002). A study on gender roles and responses to malaria in Ghana found that women who lacked either short-term or long-term economic support from male relatives, or who disagreed with husbands or family elders about seeking appropriate treatment, faced difficulties in accessing health care for children with malaria (Tolhurst and Nyonator 2005). Evidence from some countries indicates that restricted mobility of women may also impede their attendance at primary health care clinics for malaria testing. (Müller *et al*. 1998, and Lampietti *et al*. 1999).

Types of malaria

According to malaria data obtained from M&PDC during 1960's, *P. falciparum* was not the main parasite species. This species starting to get dominance since 1990's. As a consequence it contribution reached up to 94.2% of total malaria cases during 2010-13 (Figure 3.5 and 3.6). *P. vivax* was found to be involved in only 5.5% cases . However, a third malaria species *P. malariae* once had fair distribution during 60's and 70's (GoB 1977, and Khan and Talibi 1972) has now become apparently rare and involved in very few sporadic cases (Rahman *et al*. 2010) and sometimes higher in asymptomatic cases in endemic areas (Fuehrer *et al.* 2014). Recently *P. ovalae* the forth well know human malaria parasite has also been reported from Bangladesh (Fuehrer *et al.* 2010). However, till to date there is no report of the fifth human malaria parasite *P. knowlesi* in Bangladesh (Fuehrer *et al.* 2014).

In our neighboring India the relative ratio of *P. falciparum* and *P. vivax* varies greatly in different parts and often within states. Most of the Indo-Gangetic plains, northern hilly states, northwestern India and the southern Tamil Nadu state have <10% *P. falciparum* infections with the rest being *P. vivax*. The states sustaining hyperendemic malaria and the highest ratio of *P. falciparum* tend to be inhabited by ancient ethnic tribes mainly in the forest ecosystems (30–90% *P. falciparum*). Nearby fringe areas with a preponderance of *P. falciparum* (90% or even more). Other localized areas in the same state can have as low as 10 to 30% *P. falciparum* (Kumar *et al*. 2007). The representation of *P. falciparum* over *P. vivax* has been increasing in India over the last 30 years to about 60% (Kumar *et al*. 2014) which is similar to Bangladesh situation.

P. vivax is less virulent compared to *P. falciparum* but has a wide geographical distribution. About 9% of estimated cases globally are due to *P. vivax,* however its proportion outside the African continent is similar to *P. falciparum* (WHO 2013).

Malaria in Pregnancy

The rate of malaria infection is higher in pregnant women because of their decreased immunity. Studies have shown that infection rates are highest in first and second parity women with lower rates in later pregnancies. Pregnant women with malaria have an increased risk of abortion, stillbirth, premature delivery and low-birth weight

infants (Reuben *et al*. 1993, Duffy and Fried 2005). *P. falciparum* is generally accepted as a leading cause of anaemia in pregnant women (Guyatt and Snow 2001). It is estimated that anaemia causes as many as 10 000 maternal deaths each year (Steketee *et al*. 2001).

Death due to malaria

Before the introduction of the Malaria Eradication Program in 1961, the disease was so widespread that it accounted annually for 15 percent of the total deaths in Bangladesh (Robinson 1967). It has been evident that mortality due to malaria has been reduced in significantly as indicated by the present study (Table 3.1 and Figure: 3.8).

Oduro *et al.* (2007) studied severe malaria caused by the *P. falciparum* in young children of the Kassena-Nankana district of northern Ghana. The overall case fatality ratio was found 3.5%. Cerebral malaria and hyperlactataemia were the significant risk factors associated with death. Severe anaemia, though a major presentation, was not significantly associated with risk of death. In the present observation we found case fatality rate 4.0-7.3 per 1,000 malaria infected person during 2010-13 which was much lower than observed by Oduro *et al.* (2007).

Worldwide, between 2000 and 2012, estimated malaria mortality rates fell by 42% in all age groups and by 48% in children under 5 years of age. Modelling suggests that an estimated 3.3 million malaria deaths were averted between 2001 and 2012, and that 69% of these lives saved were in the 10 countries with the highest malaria burden in 2000 (WHO 2013).

Reported malaria deaths in the South-East Asia region decreased from 5500 to 1200 between 2000 and 2012. Myanmar, India and Indonesia accounted for 49%, 42% and 33% of reported deaths respectively in 2012. The reported malaria mortality rate fell by more than >75% in Bangladesh, Bhutan, Sri Lanka, Thailand and Timor-Leste between 2000 and 2012 (WHO 2013).

Malaria according to districts

Bandarban, Rangamati and Khagrachari of Chittagong Hill Tracts remain highest malria burdened district of Bangladesh (Table 3.2 and Figure 3.10). These three

districts accounted for almost 80% total malaria cases of the country on an average during 2010-13. Precisely Chittagong hill tracts contributed 78% of country's total malaria cases in 2010; 82.5% in 2011; 76% in 2012 and 80.1% in 2013 respectively (Figure 3.11). During 2009 Chittagong Hill Tracts also remain highest malaria burdened area of Bangladesh and accounted for almost 90% of country's total malaria burden (Haque *et al.* 2011).

However, during 1968-71 Chittagong, the Chittagong Hill Tracts, Sylhet, and Patuakhali were the districts with the highest level of incidence of malaria (Paul 1984). A report of the World Health Organization (WHO) classified these districts as the areas where the population of the country was most vulnerable to malaria (WHO 1977).

Except for Patuakhali, The hilly areas of Bangladesh are located in these districts. Hills and low mountains compose the Chittagong Hill Tracts, while considerable areas of hills are found in Sylhet and Chittagong. The hilly areas are mostly covered with tropical evergreen forests that provide the considerable shade needed for breeding grounds of *Anopheles.* Additional factors are part of the explanation for the high incidence of malaria during 1968-71 in Sylhet and the Chittagong Hill Tracts. Large sections of central and western Sylhet are low-lying areas locally called haor, with swampy and waterlogged terrain (Paul 1984).

Four districts Rangpur, Mymensingh, Comilla, and Barisal experienced a moderate incidence of malaria during that period (1968-71). These districts form a continuous belt located adjacent to the districts with a high incidence of disease (Paul 1984).

However during 1980s and 90s due to successful malaria control activities most of the district of Bangladesh have became free of malaria and restricted to only 13 districts which were difficult to access due to forest, hills and international boundaries (Haque *et al.* 2009a, Alam *et al.* 2010 and Haque *et al.* 2011)

Malaria species according to district

During present observation (2010-13) in three Chittagong Hill Tracts districts *P. falciparum* accounted for more than 95% of total malaria cases (Figure 3.18 and Table

3.5). Sufficient data is not available regarding district wise prevalence of malaria according to species. However, in an observation in Khagrachari during May 1966 the prevalence of *P. falciparum, P. vivax* and *P. malariae* were 50.1%, 39.0% and 10.8% respectively (Khan and Talibi 1972).

In Mymensing zone consisting of Mymensing, Sherpur and Netrokona *P. falciparum* has got the highest abundance with 96.6%, 67.8% and 92.2% of contribution of annual malaria incidences respectively. In an historical observation during April-July 1950 among the children in Mymensingh the distribution of *P. falciparum* and *P. vivax* was equal 45.5% along with 10% *P. malariae* (Quraishi *et al.* 1951).

Temporal distribution of Malaria

An increasing trend or a peak of transmission of malaria was seen in between May-October during 2010-2013 (Table 3.6 and Figure 3.20). In a time series analysis from malaria incidence of Bangladesh from 1989-2008 (Haque *et al.* 2010) found a distinct seasonality in the number of malaria cases with a peak during June to August. High temperatures occurred in April to September in each year. Except for some small fluctuations, rainfall occurred between May and October (Haque *et al.* 2010). We also found the similar trend from malaria incidence data from 2010-13.

Khan and Talibi (1972) observed the transmission of malaria with two peaks, one in the pre-monsoon period, the other in the pre-winter period in Khagrachari district. Rainfall increases the number of vector breeding places which favors malaria transmission. It is also interesting to find that the maximum number of cases occur during the months of August and September and decreases thereafter. Continuous and heavy rainfall washes off the vector breeding places. This explains the decrease in the number of malaria cases after September.

In Northern Indian states, malaria infection reports started in April onwards. Rains during June through August trigger increase in malaria positive cases during September to October. From November, vector population declines due to rainfall and temperature decrease during winter season (Dutta *et al.* 2010). In Delhi, India malaria transmission was found to occur between the months of July and September with a peak in the month of August (Kumar *et al.* 2014).

Overall trend of Malaria

A decreased overall trend for malaria transmission has been seen during 2010-13 from preceding years (2006-2009) (Figure 3.21). Malaria incidence decreased from 6·2 cases per 1000 population in 2008, to 2·1 cases per 1000 population in 2012 (Haque *et al*. 2014). According to case burden in 2013 this rate has become around 2.0 cases per 1000 population in 2013.

Globally the number of cases was estimated to have decreased from 244 million in 2005 to 207 million in 2012.The estimated number of malaria cases per 1000 persons at risk of malaria (which takes into account population growth over time) shows a reduction in case incidence of 25% globally between 2000 and 2012, and 31% in the African Region (WHO 2013).

The number of confirmed malaria cases reported in the South-East Asia region decreased from 2.9 to 2 million between 2000 and 2012. Three countries accounted for 96% of reported cases in 2012: India (52%), Myanmar (24%) and Indonesia (22%). Five countries achieved >75% decrease in case incidence between 2000 and 2012 (Bangladesh, Bhutan, Democratic People's Republic of Korea, Nepal, Sri Lanka) (WHO 2013).

The National Malaria Control Programme (NMCP) in Bangladesh has three major components in their current flagship, such as: early diagnosis by RDT (rapid diagnosis test), prompt treatment by artemisinin based combination therapy and long lasting bed net (LLIN) for vector control since 2008. The Global Fund to fight AIDS, Tuberculosis and Malaria (Global Fund) approved funds in 2006 (Round 6) and 2009 (Round 9) to support the NMCP activities which impacted hugely to reduce the malaria burden in Bangladesh.

CHAPTER 4

KNOWLEDGE, ATTITUDE AND PRACTICES (KAP) REGARDING MALARIA IN BANGLADESH

Materials and Methods

Sample size

In the current study, a total of 660 individuals (110 from each area) from three malaria endemic and three non-endemic districts of Bangladesh were enrolled for KAP study (Table 4. 1, Figure 4.1).

Data collection

The survey was conducted in portions of six different malaria endemic and non-endemic areas of Bangladesh from May 2012 to July 2014. The sample size was determined by Epi Info Version 7 statistical software. Knowledge on malaria presumed 80% as expected frequency since the area is under NMCP surveillance.

A standard structured questionnaire was designed to collect information irrespective of sex, religion and ethnicity. Interview was taken from a respondent of each household who is above or at 10 years of age. With the assistance of a local guide, middle point of each village was selected and one household in any direction of that middle point was taken as first participant. Next households were chosen with an interval of two houses to the preceding participated household.

The questionnaire was divided into several parts. The first part included socio-demographic characteristics (name, sex, age, education, occupation, number of family members, religion, tribe and physical disability, household characteristics and economic status of the household). The second part collected information regarding malaria awareness (vector, transmission, symptom and prevention) and treatment seeking and practices.

Following section was structured to collect information regarding possession and use of bed nets, age distribution among the household members. Another part included to obtain information regarding use of insecticide and possession of domestic animals.

Table 4. 1: The study areas selected for KAP survey.

Area	Area (Upazilla, District)	Number of malaria patients during 2012
Non-endemic	Savar, Dhaka	0
	Poba, Rajshahi	0
	Bagerhat, Bagerhat	0
Endemic	Matiranga, Khagrachhari	712
	Jaintiapur, Sylhet	41
	Ramu, Cox's Bazar	1545

Data analysis

Fisher's exact Chi-square test of asssociation to determine was applied to see whether there was any relationship among the socio-demographic characteristics of the respondents and prevention methods practiced between malaria non-endemic and endemic area. Multinomial logistic regression was used to model the outcome variables, in which risk ratio of the outcomes are modeled as a linear combination of the predictor variables. All data analysis has been conducted in STATA version 13 (StataCorpLP, Texas, USA).

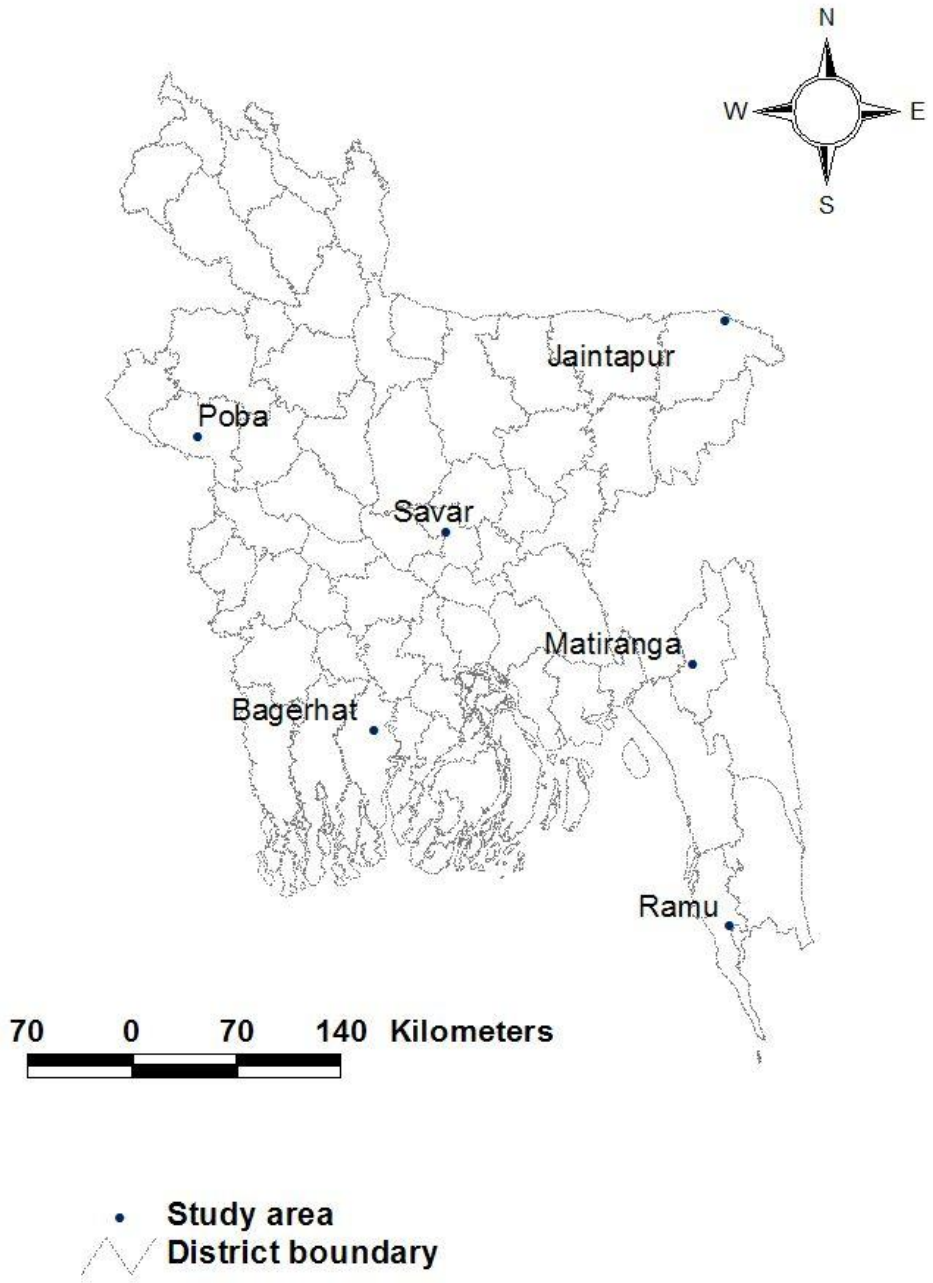


Figure 4.1: KAP study areas are shown in a Map of Bangladesh.

Results and Observations

Socio-Demographic Characteristics of the Respondents

The study enrolled 660 respondents (330 from malaria endemic areas and 330 from malaria non-endemic areas) from the same number of households. Thus one person represented one household/family.

Majority of the respondents were female (58%) where most of the respondents (29.7%) lies in the age group of 30-39 years in non-endemic area and more than 26% people in the age group of 20-29 in the endemic area. Family size was significantly smaller in non-endemic areas than endemic areas where more than 60% households possess 5 - 8 members (Table 4. 2).

In both areas, most of the respondents were married (> 82%) and deprived of any formal education (> 30% and > 50% in non-endemic and endemic region respectively). In the non-endemic area, housework became the most common occupation (30%). On the other hand, daily labor was the common (34%) means of earning livelihood in the endemic area (Table 4. 2).

Table 4.2: Demographic characteristics of the respondents in the study areas.

Characteristics	Non-endemic		Endemic		P value
	Number	%	Number	%	
a) Sex					
Male	157	47.6	115	35.0	0.001
Female	173	52.4	215	65.0	
b) Age					
≤ 19	23	7.0	37	11.2	
20-29	90	27.3	86	26.1	
30-39	98	29.7	81	24.5	0.006
40-49	38	11.5	65	19.7	
50-59	42	12.7	38	11.5	
≥ 60	39	11.8	23	7.0	
c) Marital Status					
Unmarried	31	9.4	46	13.9	
Married	295	89.4	272	82.4	0.044
Widower/Widow	4	1.2	11	3.3	
Divorced	0	0	1	0.3	
d) Education					
No Formal Education	110	33.3	178	53.9	
Primary	80	24.2	76	23.0	0.000
SSC	101	30.6	60	18.2	
HSC	23	7.0	10	3.0	
Graduate	11	3.3	4	1.2	
Master	5	1.5	2	0.6	
e) Number of Family Members					
≤ 4	183	55.5	97	29.4	
5-8	121	36.7	210	63.6	0.000
≥ 9	26	5.8	23	7.0	

Table 4.2 (contd…): Demographic characteristics of the respondents in the study areas.

Characteristics	Non-endemic		Endemic		P value
	Number	%	Number	%	
f) Occupation					
Farmer	47	14.2	59	17.9	
Day Laborer	69	20.9	112	33.9	
Service holder	28	8.5	19	5.8	
Businessman	45	13.6	17	5.2	
Technician	1	0.3	5	1.5	0.000
House Worker	99	30.0	78	23.6	
Student	12	3.6	24	7.3	
Motor Driver	5	1.5	2	0.6	
Expatriate	0	0	1	0.3	
Beggar	1	0.3	2	0.6	
Unemployed	23	7.0	11	3.3	
g) Tribe					
Bengali	330	100	303	91.8	0.000
Tripura	0	0	27	8.2	
h) Religion					
Islam	329	99.7	320	97.0	0.017
Hindu/Shanatan	1	0.3	10	3.0	

Knowledge on malaria and awareness

The term 'Malaria' was well known to the community. More than 50% respondents of endemic area reported that they had experienced malaria among the household members and 24.5% of them said that there was at least one malaria patients in their house within a year. Number of infections in the past five years between non-endemic and endemic areas was significantly different ($p < 0.05$) (Table 4. 3).

Mosquito bite is responsible for malaria was known to more than 60% of the respondents from both areas. Where malaria transmission was not well known in the non-endemic area but 66% respondents of endemic area reported that people get malaria by mosquito bite. However, the cause of malaria transmission that a female infected anopheline transmits the disease remained vague in both areas (Table 4. 3). More than 70% of the respondents didn't know the symptoms of malaria in the non-endemic area whereas; 'onset of fever with shivering' was the common answer among the respondents of the endemic area. Significant ($p < 0.05$) difference was observed among the responses of two areas regarding the symptom of malaria (Table 4. 3).

In both areas, people seemed to rely mostly on bed nets (more than 40%) to prevent malaria and if they become infected, allopathic medicine was the choice of treatment (about 90%). The treatment seeking behavior varied significantly ($p < 0.05$) between the respondents of the two areas. Government hospitals were chosen by most (41%) of the inhabitants of non-endemic area whereas, private health center (> 30%) and village doctor (> 30%) were preferred by the respondents of endemic area. Non-government health workers seemed to play key role of circulating malaria awareness in endemic area (Table 4. 3).

Table 4. 3: Knowledge, attitude, and practice regarding malaria among the respondents.

Characteristics	Non-endemic		Endemic		P value
	Number	%	Number	%	
a) Heard about malaria					
Yes	317	96.1	325	98.5	0.056
No	13	3.9	5	1.5	
b) Any of the member had malaria within the past five years					
Yes	4	1.2	168	50.9	0.000
No	326	98.8	162	49.1	
c) If yes, how many days ago					
Less than one month	0	0	23	7.0	
Less than three months	0	0	29	8.8	0.000
More than six months	0	0	36	10.9	
More than one year	4	1.2	81	24.5	
No malaria	326	98.8	161	48.8	
d) Why someone gets malaria					
Do not know	99	30.0	66	20.0	
Mosquito bites	211	63.9	219	66.4	0.002
Fly/insects bites	2	0.6	4	1.2	
Lack of cleanliness	18	5.5	39	11.8	
Airborne	0	0	2	0.6	
e) Transmission of malaria					
Do not know	211	63.9	100	30.3	
Bitten by any mosquito	87	26.4	194	58.8	0.000
Bitten by a female anopheline mosquito which had already bitten a malaria patient	31	9.4	33	10.0	
Due to infection	1	0.3	3	0.9	
f) Symptoms of malaria					
Do not know	232	70.3	83	25.2	
Onset of fever with shivering	88	26.7	230	69.7	0.000
Fever at intervals	10	3.0	9	2.7	
Remission of fever with sweating	0	0	8	2.4	

Table 4. 3 (contd…): Knowledge, attitude, and practice regarding malaria among the respondents.

Characteristics	Non-endemic		Endemic		P value
	Number	%	Number	%	
g) Prevention					
Do not know	103	31..2	69	20.9	
Limiting mosquitoes' oviposition sites	82	24.8	73	22.1	0.001
Bed net	132	40.0	170	51.5	
Mosquito coil	13	3.9	9	2.7	
ITN/LLIN	0	0	6	1.8	
Taking medicine	0	0	3	0.9	
h) Treatment					
Do not know	6	1.8	27	8.2	
Allopathic	316	95.8	285	86.4	0.000
Kabiraji/unani	1	0.3	1	0.3	
Spiritual	0	0	12	3.6	
homeopathic	7	2.1	5	1.5	
i) Where treated					
Do not know	3	0.9	30	9.1	
Government hospital	135	40.9	63	19.1	0.000
Private health center	54	16.4	111	33.6	
Village doctor	49	14.8	111	33.6	
Drug seller	89	27.0	15	4.5	
j) Source of information					
Government health worker	64	19.4	45	13.6	0.000
Non-government health worker	33	10.0	183	55.5	
Radio	6	1.8	14	4.2	
Newspaper	4	1.2	2	0.6	
Advertising board	83	25.2	2	0.6	
Poster	4	1.2	20	6.1	
Book	3	0.9	8	2.4	
N/A	133	40.3	56	17.0	

Use and possession of bed nets

Almost all the respondents were explicitly reported to have at least one bed net (> 98%) despite a tiny fraction who did not have any bed net. In the endemic area nearly 80% of the respondents reported that they use bed nets regularly and most of the respondents believed that use of bed nets can prevent malaria. However, still a high percentage of respondents (more than 50%) contracted malaria. Surprisingly more than 80% of the respondents from the endemic area reported that the ITNs they were supplied were not treated within the last six months of the present (Table 4. 4).

Table 4.4: Percentage of using the bed nets among the respondents in the study areas.

Characteristics	Non-endemic		Endemic		P value
	Number	%	Number	%	
a) Possession of bed net					
Yes	325	98.5	324	98.2	0.056
No	5	1.5	6	1.8	
b) Use of bed nets					
Summer	8	2.4	11	3.3	0.026
Winter	29	8.8	14	4.2	
All the year	288	87.3	293	88.8	
Do not use	0	0	5	1.5	
N/A	5	1.5	7	2.1	
c) Sleeping under the bed net regularly					
Yes	272	82.4	261	79.1	0.000
No	25	7.6	59	17.9	
Seldom/irregularly	28	8.5	4	1.2	
N/A	5	1.5	6	1.8	
d) Bed net treated within last 6 months					
Yes	0	0	47	14.2	0.000
No	330	100	283	85.8	

Use of insecticide and possession of domestic animal

More than half of the respondents use any measure for mosquito control and mosquito coil became the commonest tool. However, approximately 35% of the respondents in both areas use mosquito coil if they feel required. Possession of domestic animal is common in both areas (Table 4. 5).

Table 4. 5: Collected data on the use of insecticide and possession of domestic animal of the respondents.

Characteristics	Non-endemic		Endemic		P value
	Number	%	Number	%	
a) Use anything for mosquito control					
Yes	186	56.4	166	50.3	0.119
No	144	43.6	164	49.7	
b) Kind of insecticide					
Mosquito coil	172	52.1	138	41.8	0.037
Dhup	2	0.6	4	1.2	
Smoke	10	3.0	22	6.7	
Electric device	2	0.6	3	0.9	
N/A	144	43.6	164	49.7	
c) How often the insecticides are used					0.060
Always	49	14.8	35	10.6	
Sometime	115	34.8	120	36.4	
Only in summer months	22	6.7	11	3.3	
N/A	144	43.6	164	49.7	
d) Cost of insecticide					
None	0	0	10	3.0	0.000
< BDT 100	141	42.7	109	33.0	
< BDT 200	14	4.2	20	6.1	
> BDT 200	31	9.4	13	3.9	
N/A	144	43.6	178	53.9	
e) Possession of domestic animal					
Yes	193	58.5	207	62.7	0.265
No	137	41.5	123	37.3	

Household economic characteristics of the respondents' and practices

Most of the households were observed to be 'Not Jhupri' i.e. permanent/semi permanent structure. Construction of household partition mostly done by mud (51%) in endemic areas which is significantly ($p < 0.05$) different from households in non-endemic areas where household partitions were mostly cemented (55%). Mud floor was also common is endemic areas (84%), where in non-endemic areas mud and cemented floors are both equally popular. Significant ($p < 0.05$) difference in terms of economic status has been observed between non-endemic and endemic areas. Nearly 50% of the respondents from non-endemic areas reported that their income is surplus, on the other hand more than 30% respondents from endemic areas reported that their monthly income is sometime deficient (Table 4. 6). Although most respondents from both areas reported that their economic condition has not been improved within the past five years, respondents of the endemic areas seemed to pass more deteriorated economic change in last five years (Table 4. 6).

Nearly equal number of respondents (about 58%) spent their after noon time at outside in both endemic and non-endemic areas (Table 4. 6). It has been observed that most of the respondents (about 70%) enter during 6 to 10 pm in the house of both areas (Table 4. 6).

Table 4. 6: Household characteristics of the respondents in the study areas.

Characteristics	Non endemic		Endemic		P value
	Number	%	Number	%	
a) Roof of the house					
Straw/thatch	15	4.5	49	14.8	0.000
Tin	281	85.2	277	83.9	
Concrete/cement	34	10.3	4	1.2	
b) House partition					
Jute stick/bamboo	39	11.8	82	24.8	0.000
Tin	37	11.2	34	10.3	
Concrete/cement	184	55.8	38	11.5	
Mud	60	18.2	168	50.9	
Wood	9	2.7	8	2.4	
Plastic	1	0.3	0	0	
c) Floor					
Mud	163	49.4	278	84.2	0.000
Cemented	152	46.1	50	15.2	
Semi-cemented	15	4.5	0	0	
Wood	0	0	2	0.6	
d) House status					
Jhupri	23	7.0	40	12.1	0.024
Not jhupri	307	93.0	290	87.9	
e) Monthly income of the family					
< BDT 5000	51	15.5	155	47.0	0.000
> BDT 5000 – 10000	158	47.9	135	40.9	
> BDT 10000 – 15000	70	21.2	18	5.5	
> BDT 15000 – 20000	26	7.9	10	3.0	
> BDT 20000	25	7.6	12	3.6	

Table 4. 6 (contd…): Household characteristics of the respondents in the study areas.

Characteristics	Non endemic		Endemic		P value
	Number	%	Number	%	
e) Economic status					
All the year deficient	40	12.1	94	28.5	0.000
Deficient sometimes	76	23.0	104	31.5	
Not deficient nor surplus	54	16.4	73	22.1	
Surplus	160	48.5	59	17.9	
f) Economic change in past five years					
Condition improve	131	39.7	41	12.4	0.000
Not improved	191	57.9	251	76.1	
Condition deteriorated	8	2.4	38	11.5	
g) Spent time in the afternoon hours					
Inside	121	36.7	102	30.9	0.057
Outside	190	57.6	191	57.9	
Market	12	3.6	25	7.6	
Tea Stall	7	2.1	12	3.6	
h) Usually enter the house					
< 6 pm	73	22.1	103	31.2	0.000
6-10 pm	228	69.1	208	63	
> 10 pm	29	8.8	19	5.8	

Estimation of relative risk factors

Transmission of malaria has been observed to be significantly ($p < 0.05$) related with the educational status. Respondents who are graduate or equal educational qualification had two times more knowledge on the malaria transmission related information compare to people who had no formal education (Table 4. 7). People of the endemic areas who had completed master education knew 1.3 times more about the cause of malaria than people of the non-endemic area (Table 4. 8). On the other hand, people who undertook graduation was observed to know 1.5 times more about the malaria symptoms (Table 4. 9). Knowledge on the malaria transmission has been possessed more than 2 times by the families who earn more than BDT 20000 comparing with those families who earn BDT 5000 (Table 4. 10). Use of insecticides for mosquito control has observed to be significantly related with the monthly income of the family. Families whose monthly income exceeds BDT 20000 per month use insecticides 3 times more than the base group (Table 4. 11). Likewise, possession of bed nets has observed to be strongly associated with the wealth of the family. More bed nets have also been significantly possessed by the families of more monthly income (Table 4. 12). However, families whose income limited to BDT 5000 and > BDT 5000 – 10000 significantly answered that they had malaria patients in the past 5 years, which was more than 2 and 3 times respectively than families earning > BDT 20000 (Table 4. 13).

Table 4. 7: Relationship among different education groups regarding the knowledge of malaria transmission.

Education Group	Relative Risk Ratio	P value	95% Confidence Interval	
Primary Education	0.93	0.648	.4348485	.7314892
SSC	1.2	0.206	0.3806947	0.649634
HSC	1.99	0.006	.0384015	.1201768
Graduate	2.21	0.024	.0118918	.0653227
Master	2.21	0.116	.0037884	.0446554

Reference variable: No Formal Education

Table 4. 8: Relationship among different education groups and the knowledge on the cause of malaria.

Education Group	Relative Risk Ratio	P value	95% Confidence Interval	
Primary Education	0.98	0.934	.4046025	.7395088
SSC	1.12	0.364	.3721545	.6793333
HSC	1.23	0.320	.052526	.1638745
Graduate	0.92	0.807	.0253344	.1250021
Master	1.37	0.450	.0052828	.0583145

Reference variable: No Formal Education

Table 4. 9: Relationship among different education groups and the knowledge on the symptom of malaria.

Education Group	Relative Risk Ratio	P value	95% Confidence Interval	
Primary Education	0.83	0.280	.4594447	.7830674
SSC	0.78	0.140	.4932179	.8332587
HSC	1.16	0.586	.0619999	.1738387
Graduate	1.56	0.238	.0174827	.0833347
Master	1.30	0.643	.0068278	.0610444

Reference variable: No Formal Education

Table 4. 10: Relationship among monthly family incomes and the knowledge on the transmission of malaria.

Monthly income of the family	Relative Risk Ratio	P value	95% Confidence Interval	
> BDT 5000 – 10000	1.02	0.831	1.095056	1.781646
> BDT 10000 – 15000	0.69	0.078	.3758957	.7179525
> BDT 15000 – 20000	1.94	0.008	.0598392	.1835592
> BDT 20000	2.14	0.002	.0554561	.1729734

Reference variable: Monthly family income BDT 5000

Table 4. 11: Relationship among monthly family income and the use of insecticides of mosquito control.

Monthly income of the family	Relative Risk Ratio	P value	95% Confidence Interval	
> BDT 5000 – 10000	0.72	0.068	1.302125	2.169731
> BDT 10000 – 15000	1.21	0.456	.2608189	.5623547
> BDT 15000 – 20000	1.68	0.173	.0700034	.2328024
> BDT 20000	3.04	0.009	.0413497	.1751669

Reference variable: Monthly family income BDT 5000

Table 4. 12: Relationship among monthly family income and the possession of bed nets.

Monthly income of the family	Relative Risk Ratio	P value	95% Confidence Interval	
> BDT 5000 – 10000	1.46	0.000	.4233984	.9842974
> BDT 10000 – 15000	2.44	0.000	.0268238	.1002987
> BDT 15000 – 20000	3.45	0.000	.0023728	.0184494
> BDT 20000	3.37	0.000	.0028337	.0209745

Reference variable: Monthly family income BDT 5000

Table 4. 13: Relationship among monthly family income and the occurrence of malaria in the last 5 years.

Monthly income of the family	Relative Risk Ratio	P value	95% Confidence Interval	
BDT 5000	2.63	0.055	3.112121	6.688817
> BDT 5000 – 10000	3.07	0.024	4.259302	8.988599
> BDT 10000 – 15000	0.38	0.152	1.725076	3.899851
> BDT 15000 – 20000	1.54	0.496	.5482941	1.497899

Reference variable: Monthly family income BDT 20000

Comparison of demographic characteristics between non-endemic and endemic area

'Daily labor' has been observed to be the most common means of livelihood in endemic area whereas, housework has been the most common occupation for the respondents for non-endemic area (Figure 4. 2). Most of the people in endemic area do not have any formal education. However, all education groups in the endemic area outnumbered by the respondents of non-endemic area (Figure 4. 3). Concrete or cemented household partition observed to be common in non-endemic area. However, mud constructed walls was most common in endemic area (Figure 4. 3).

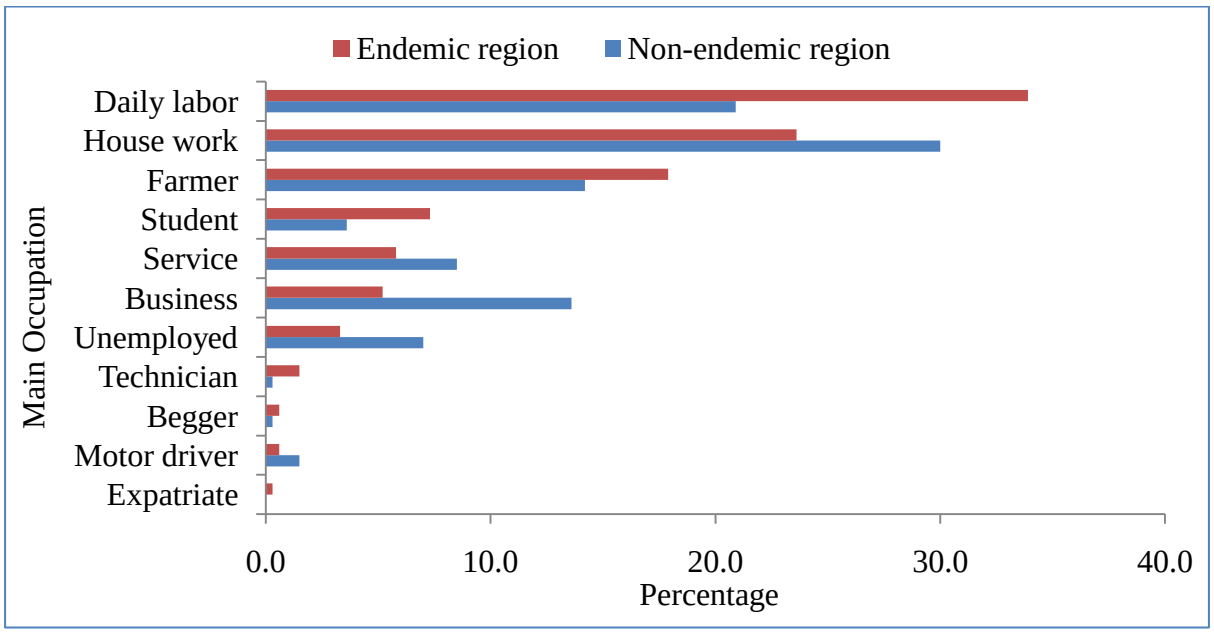


Figure 4. 2: Comparison of the occupation among the respondents of non-endemic and endemic areas.

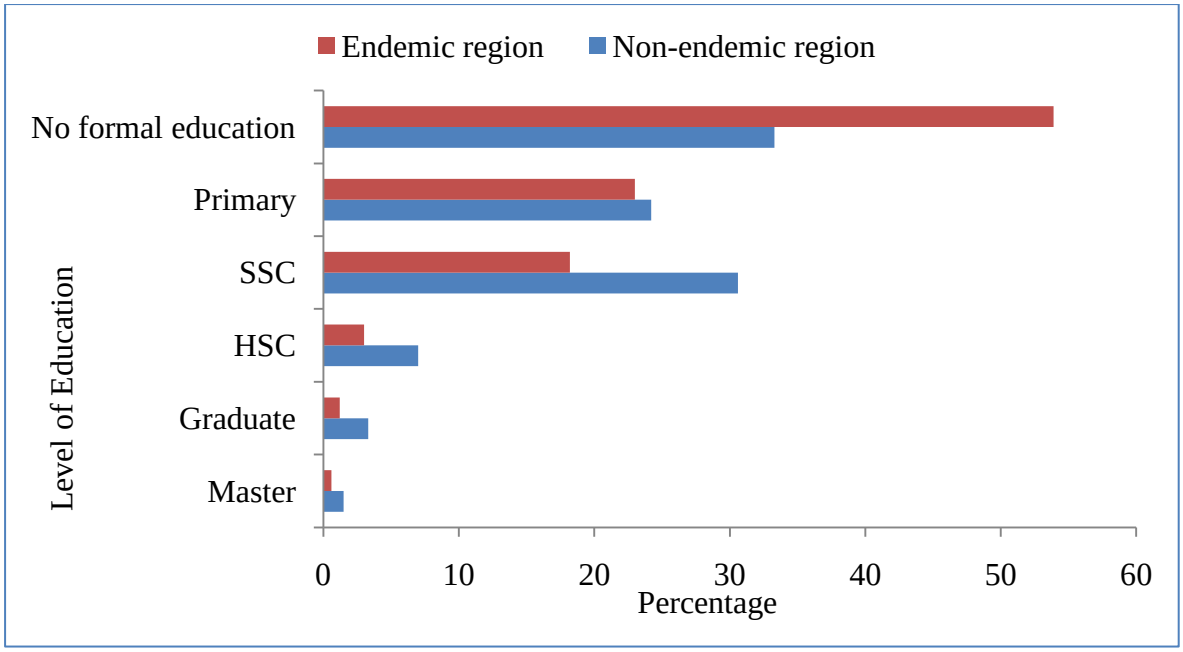


Figure 4. 3: Comparison of the level of education among the respondents of non-endemic and endemic areas.

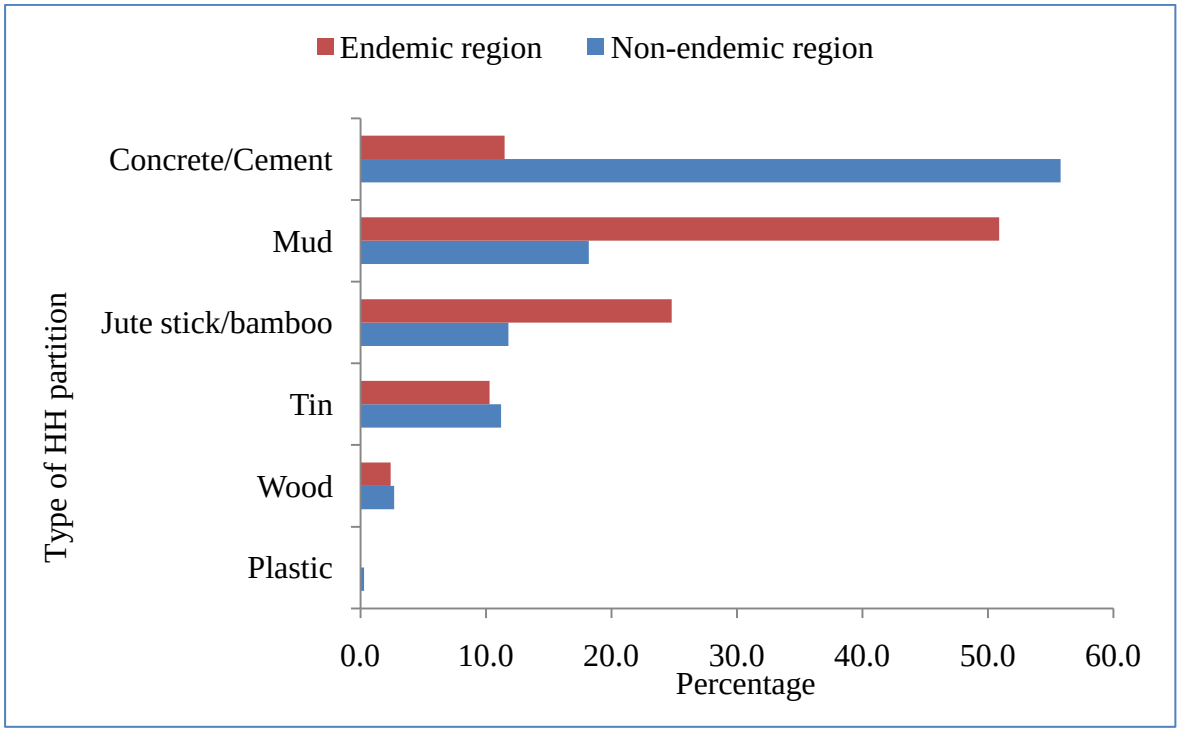


Figure 4. 4: Comparison of the type of partitions among the households of non-endemic and endemic areas

Comparison of general practices and knowledge among the respondents between non-endemic and endemic areas

Equal number of respondents reported to reside outside of the house at the evening time. However, market places and tea stalls seemed to be the popular places to spend time for the respondents of endemic area (Figure 4. 5). Although equal number of respondents knew 'mosquito bite' is responsible for malaria (about 65%) (Figure 4. 6) but number of malaria cases in past 5 years were significantly common in endemic areas among the respondents (> 50%) (Figure 4. 7). Where the knowledge 'bitten by any mosquito' can cause malaria was significantly common among the respondents of endemic area than non-endemic area, the actual cause of transmission that 'bitten by an infected mosquito' was known to few respondents (Figure 4. 8). Regarding the symptom of malaria, respondents of the endemic areas significantly answered correctly than the respondents of the non-endemic area that onset of fever with rigor is the common symptom malaria (about 70%) (Figure 4. 9). Interestingly, use of bed net to prevent malaria answered by more responded of the endemic area than non-endemic area. This might be the cause of the distribution of ITNs among them (Figure 4. 10). Allopathic treatment seemed to be the most common (> 86%) means of treatment in both areas where the respondents of non-endemic areas preferred to go to government hospitals but private health center and village doctor were equally popular (33.6% for both answer) for the inhabitants of endemic area (Figure 4. 11). A significant relationship has been observed between the use of insecticide and economic status. Respondents of the non-endemic areas reported to use more mosquito coil (> 52%) than the respondents of the endemic areas (Figure 4. 12). The most common source of malaria information was observed to be obtained from non government health workers in the endemic areas (Figure 4. 13).

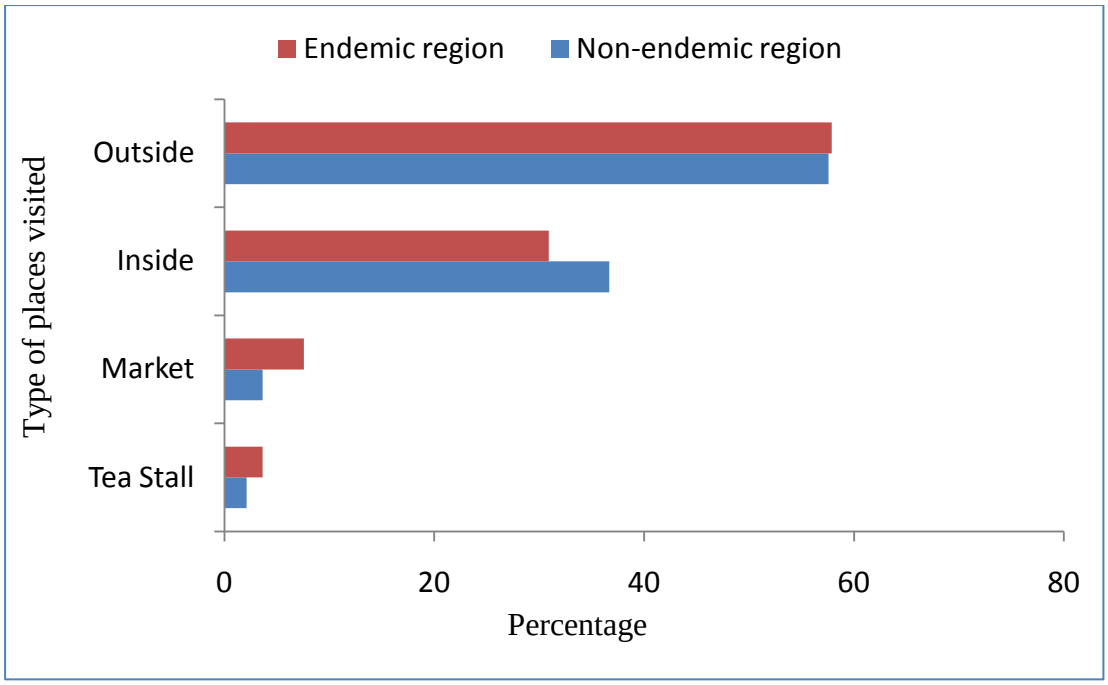


Figure 4. 5: Type of places visited by the respondents of non-endemic and endemic areas at evening time.

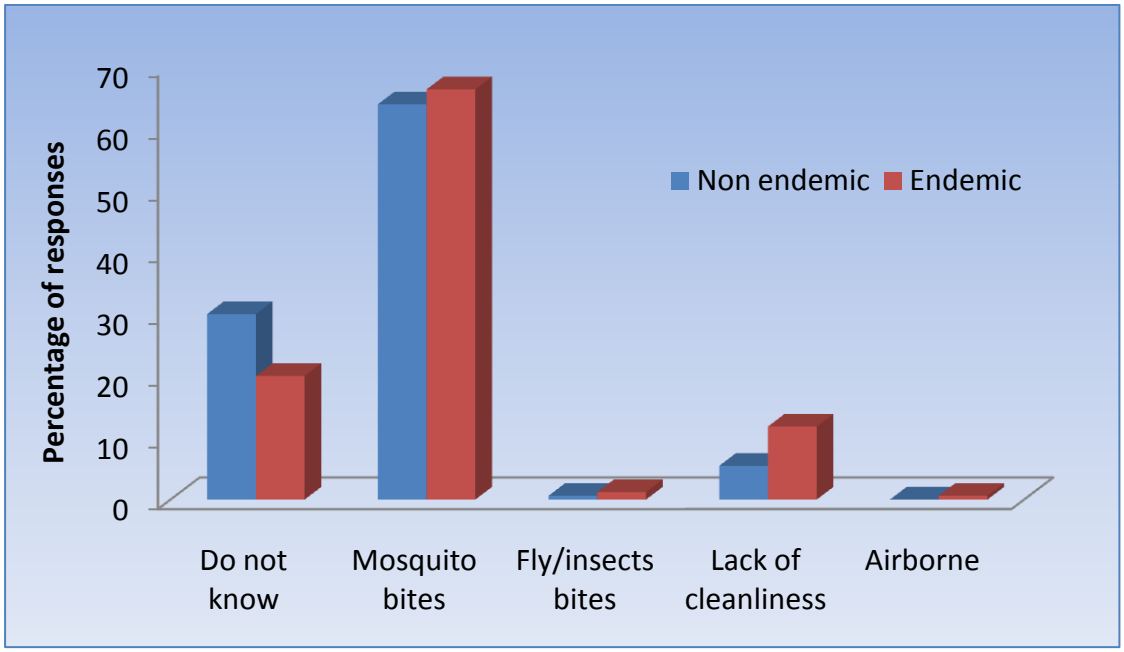


Figure 4. 6: The knowledge regarding the cause of malaria among the respondents of non-endemic and endemic areas.

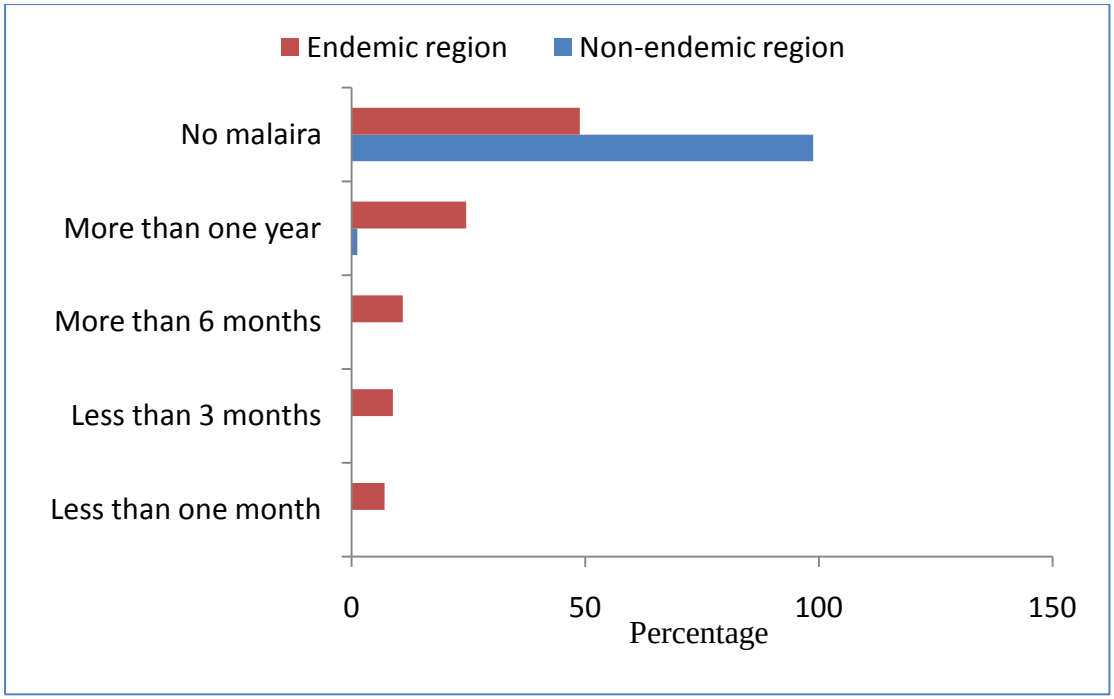


Figure 4. 7: The incidence of malaria in different times among the respondents of non-endemic and endemic areas.

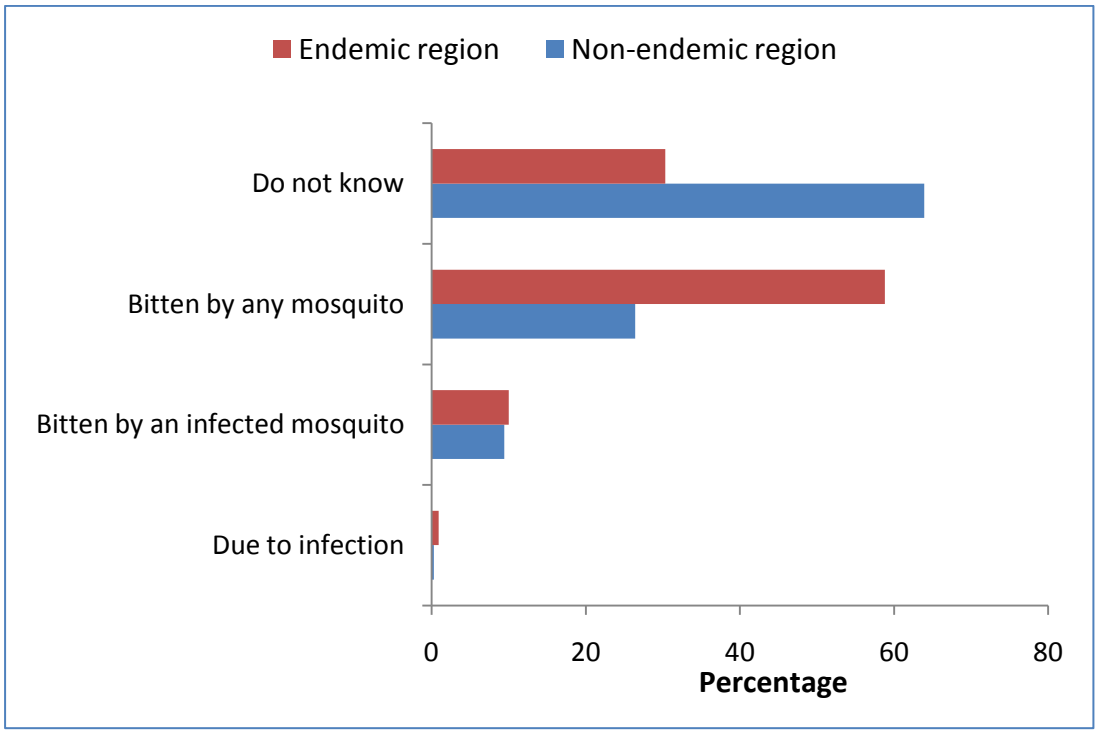


Figure 4. 8: The knowledge of malaria transmission among the respondents of non-endemic and endemic areas.

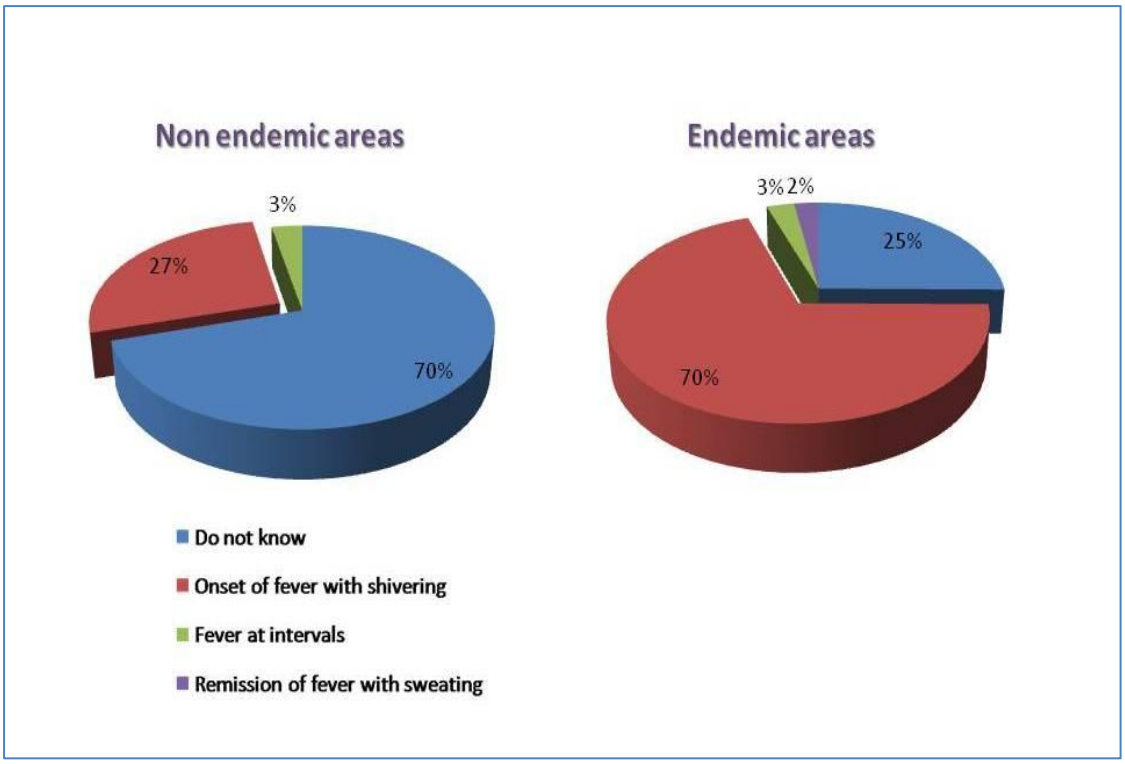


Figure 4. 9: The knowledge regarding malaria symptoms among the respondents of non-endemic and endemic areas.

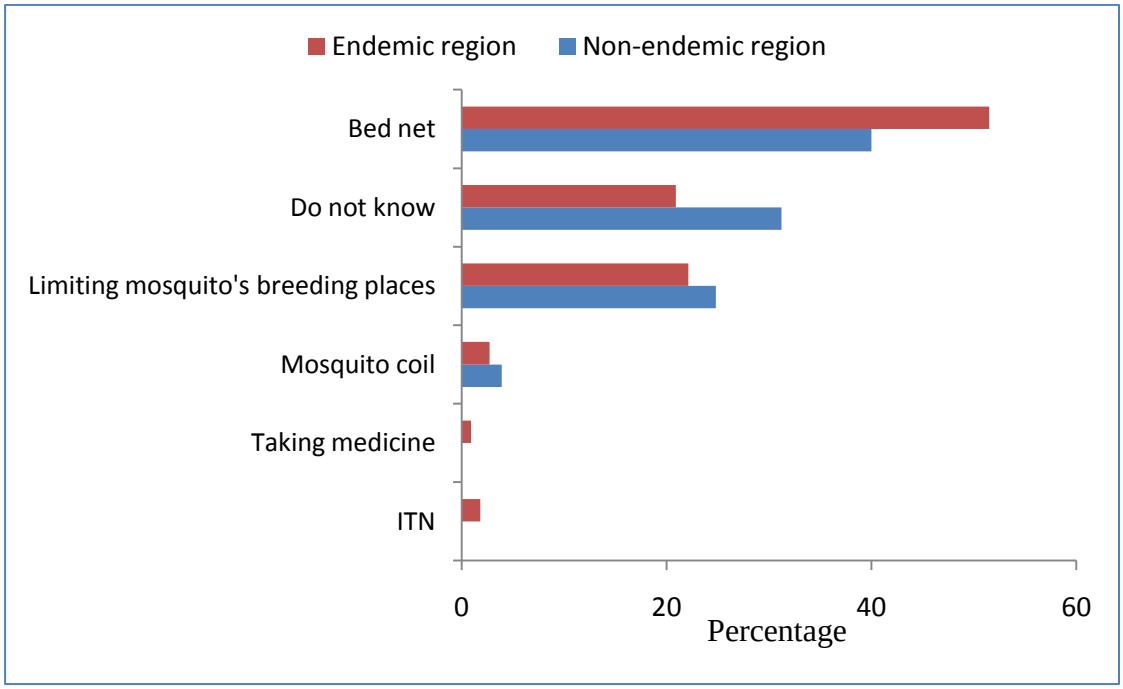


Figure 4. 10: The knowledge regarding malaria prevention among the respondents of non-endemic and endemic areas.

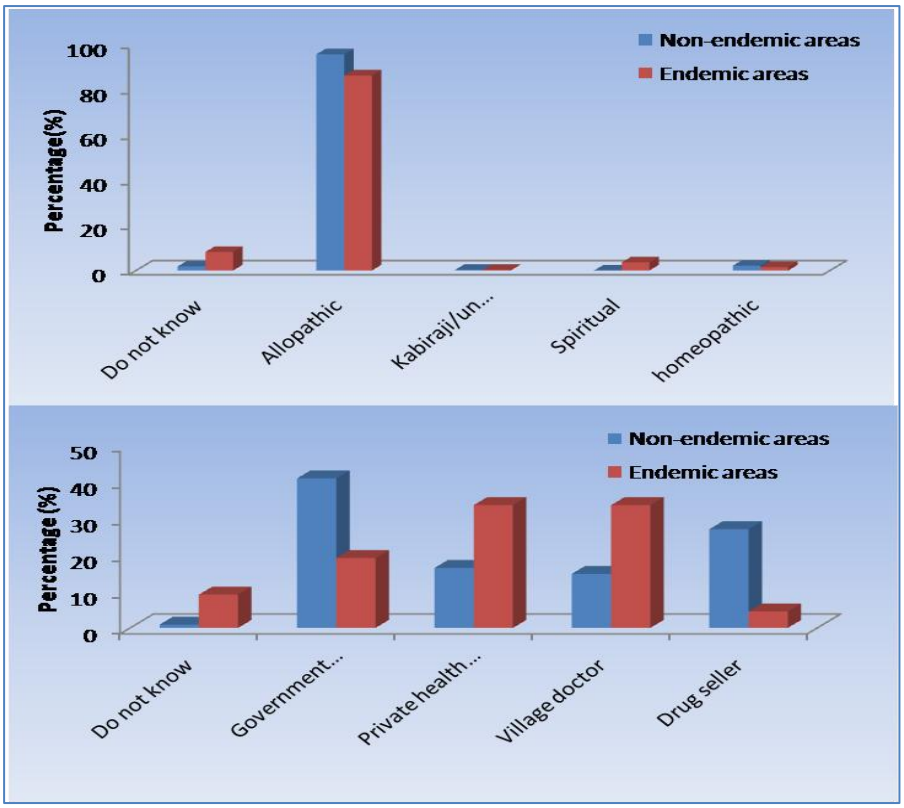


Figure 4. 11: The preferred treatment method by the respondents in non-endemic and endemic areas.

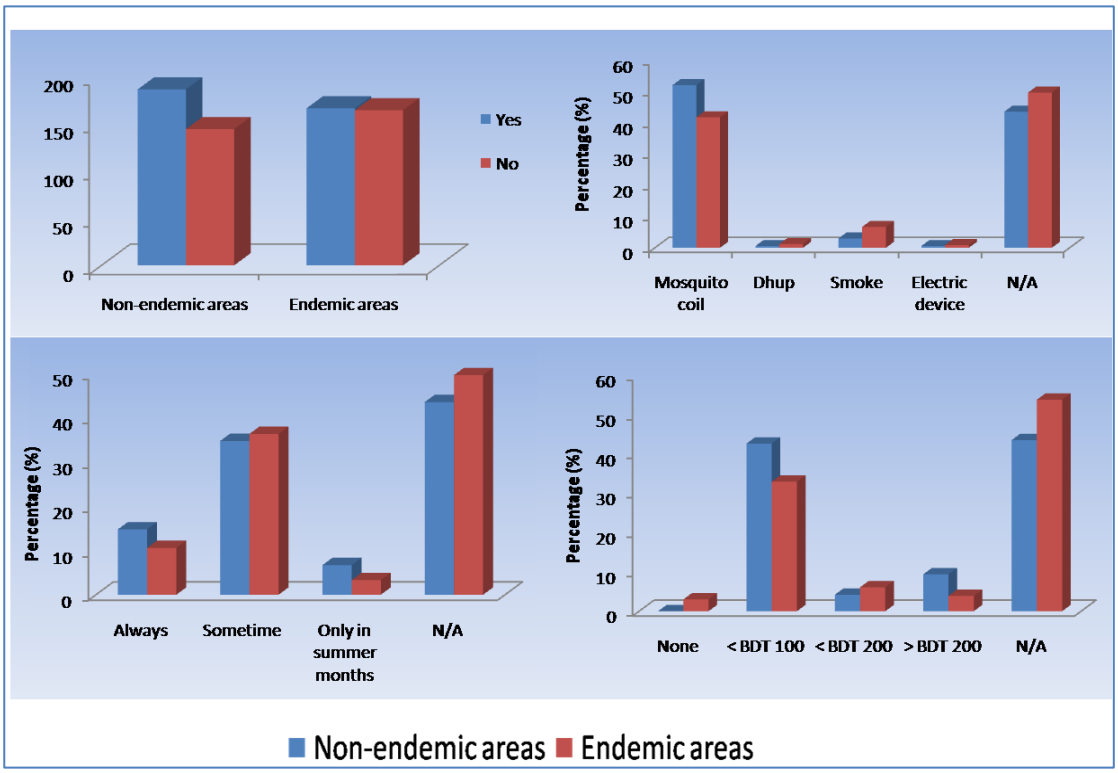


Figure 4.12: The type of insecticides used by the respondents of non-endemic and endemic areas.

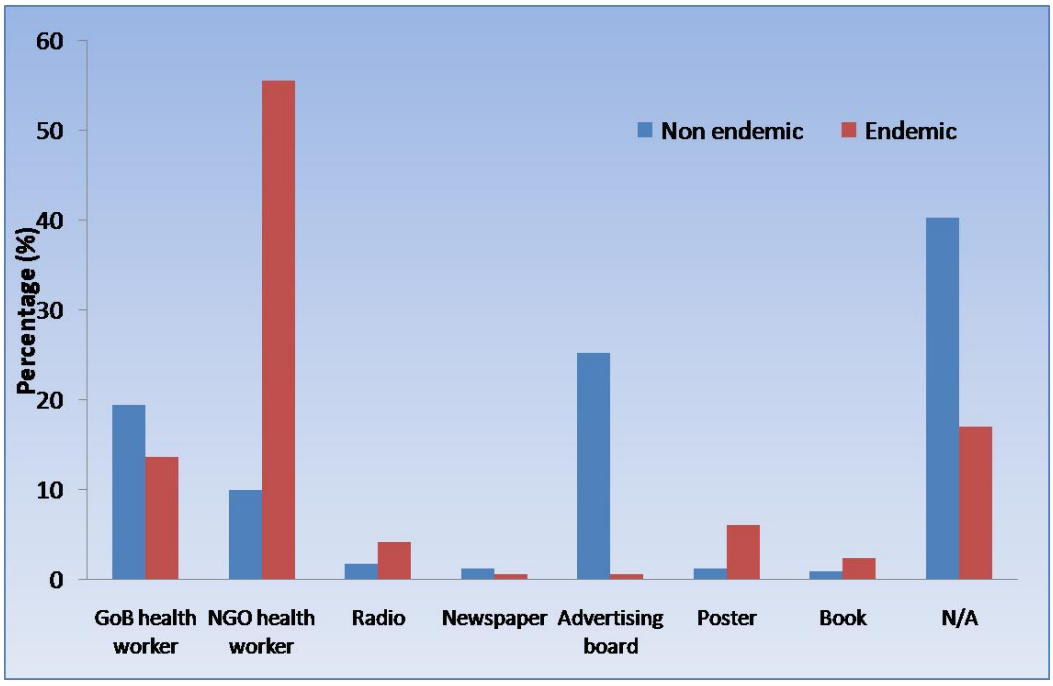


Figure 4. 13: The sources of malaria related information obtained by the respondents in the study areas.

Discussion

The potential contribution of KAP studies to malaria research and control has not received much attention in most countries of the world including Bangladesh (Ahmed *et al.* 2009, Hlongwana *et al.* 2009). The role of KAP research in the design and implementation of evidence-based prevention, management and control strategies for malaria is very important (Mwenesi, 2005). The present study attempted to address the current knowledge gaps in malaria by presenting data on malaria knowledge, attitude and practices.

Socio-Demographic Characteristics of the Respondents

Majority of the respondents enrolled in the present study are female. Since in Bangladesh men are mostly involved in outdoor activities, women reside in the house and engage in household works therefore are more available to participate in the study. Similar situation also reported in a recent study in Bangladesh (Bashar *et al.* 2012). Most of the people in the present are deprived of education, as it remains the general education situation in Bangladesh. Joint families are frequently seen in the villages, that is why most of the households in the present possessed by 5 to 8 persons. Daily wage for earning livelihood has been observed to be the most common means in endemic area that has also been significantly different from the non-endemic area. In the endemic area most of the peoples' income is limited within BDT 5000 and they did not enjoy any favorable economic change in the past five years.

Malaria Awareness and Knowledge

Community knowledge on malaria prevention and control options are important and the effort is related to personal protection, management, and control of vectors. It is revealed from the current study that around 65% people in both areas acquire malaria by mosquito bites but only about 10% people knew that a female anopheline mosquito when had bitten an malaria infected person become infected and can transmit malaria. This is consistent with previous KAP studies in Bangladesh and other countries. For instance Ahmed *et al.* (2009) (>90%), Bashar *et al.* (2012) (about 70%), Okello-Ogojo (2001) (about 74%) and Singh *et al.* (2014) (74%) revealed the knowledge on mosquito bites in transmission of malaria. Recognition of early malaria symptom is very important to get rapid treatment. However, in the endemic areas 25% people did not

know the symptoms and situation seemed to be worse in non-endemic areas where more than 70% people did not know the symptoms. This is mostly due to fewer awareness programs in the non-endemic areas. On the other hand, 'onset of fever with shivering' was known to nearly 70% people in the endemic areas reported in the present study is consistent with other reports (Joshi and Banjara 2008, Simsek and Kurcer 2005, Jima *et al.* 2005, Rakhshani *et al.* 2003 and Deressa *et al.* 2004).

In Ethiopia, it was found that more than 80% respondent knew about malaria symptoms. 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. Peoples' knowledge was found quite high. 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 (Deressa *et al*. 2004).
It has seen in the present study that people rely mostly on bed nets as a preventive measure against mosquito bite. Similar high level of knowledge on preventive use of bed net was also observed from Bangladesh (Ahmed *et al.* 2009), Nepal (Joshi and Banjara, 2008) and Ghana (De La Cruz *et al.* 2006) but lower at Ethiopia (Jima *et al.* 2005), Iran (Rakhshani *et al.* 2003), Delhi (Tyagi *et al.* 2005) and Turkey (Simsek and Kurcer, 2005). This will make control programs' and interventions' in the endemic areas easy to introduce bed nets and ITNs (Insecticide Treated Nets). However, programs needs to keep the equity perspective in focus while distributing ITNs or treating the supplied ITNs with insecticide because the poorer were disadvantaged in bed possession or re-treatment of ITNs as observed in the present study.

Respondents would highly accept modern allopathic treatment for malaria. Whether formal or informal way they would seek treatment from village doctors or private health centers as revealed from the current study. Unlike to the studies of Turkey (Simsek and Kurcer, 2005) self-treatment practice for malaria was not common in Bangladesh. However, the health care providing personnel, who are sought for curative treatment is not believed to be well qualified and their treatment is not without question (Ahmed and Hossain, 2007). Local drug sellers are in close contacts with the villagers therefore, significantly associated with the choice of treatment. Nearly 50% of the malaria treatment facility was provided by local drug sellers because (Haque *et al.*

2010). From the outcomes of the current study, almost all of the respondents were familiar with at least some aspects of malaria. Non-government health workers played a key role for providing information and it seems to be that they maintain contact with the villagers. Since more than 30% people of the endemic area did not possess the proper knowledge of transmission of malaria and non-government health workers are well ahead of providing information, government should concentrate more on educating people on malaria.

Use and possession of bed nets

Current malaria control strategy in Bangladesh mainly relies on the use of ITNs. For several years this strategy has benefited from mass campaign promotions (Ahmed *et al.* 2011). Before the introduction of ITNs, there were about 50,000 confirmed cases and around 500 annual deaths due to malaria (Ahmed *et al.* 2011). However, this mortality dropped down to less than 50 in 2010 after the ITNs distribution (World Health, 2013, Ahmed *et al.* 2011). Free distribution of ITNs in other countries also benefitted people from malaria infection and lowered the morbidity (Aderaw, 2013). In the current study nearly all the households possessed bed nets and most of them believe bed nets can prevent malaria. Similar situated has also been observed in the previous studies in Bangladesh (Ahmed *et al*. 2009, Bashar *et al.* 2012) and India (Tyagi *et al*. 2005).

Use of insecticide and possession of domestic animal

Use of insecticide for mosquito control methodology reflects the socio-economic condition of the respondents (Banguero, 1984). In the present, high usage of commercially available mosquito coils by the respondents of non-endemic areas and low in endemic areas explained the impact of socio-economic conditions on the selection of protection means in communities. Soan and Chand (2006) found smoke was the major preventive measure taken up by the respondents to avoid mosquito bite in a tribal community in India. 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. However, in the present study we found high percentage of bed net use in our study areas.

It has been well established that if mosquito blood meal could be diverted towards wild or domestic animals, which are not the reservoir hosts of malaria, the number of would be reduced (WHO 1982). This is in contrast with the present study where people who kept animals had malaria. This observation was also supports the results of a study in Pakistan (Idrees and Jan 2001). They reported that malaria parasite rate were greater among the children of families who kept cattle than who did not. Recent studies detected *Plasmodium* parasites from some *Anopheles* species which were previously believed to be zoophilic (Alam *et al.* 2010, Alam *et al.* 2012). Thus some of these zoophilic mosquitoes may feed upon humans and could potentially transmit malaria (Alam *et al.* 2012).

Household economic characteristics of the respondents' and practices

Economics played a key role in malaria in the present. For instance, poor families had a greater percentage of malaria and this was likely influenced by construction materials used. As has been reported by others (Gamage-Mendis *et al*. 1991), in malaria endemic areas the risk of infection is higher in poorly built houses than in well-built ones. in southern Sri Lanka, the risk of malaria was reported to be 2.5 times more for residents of poorly constructed houses compared to people living in houses of better construction type (Gunawardena *et al*. 1998). It is evident from other studies that mud house is preferred by mosquitoes (Kirby *et al*. 2008). In the current study, similar situation has been observed between malaria prevalence and household types were in endemic area.

Malaria prevalence was also linked to family size such that large families sharing a single household tended to have higher prevalence of malaria. This relationship might be related to the fact that large family households contain people who are generally poor, deprived of general education and living in poorly built houses. In addition, many of the members spent time outdoors during late afternoon or early evening when come at house from work and due limited accommodation of the main living room and shortage of bed nets many of them did sleep under a bed net (Bashar *et al*. 2012).

CHAPTER 5

ESTABLISHMENT AND EVALUATION OF REAL-TIME PCR FOR *PLASMODIUM FALCIPARUM*

Materials and Methods

Sampling area

The study samples were collected from Matiranga Upazila (sub-district) of Khagrachari district situated at the south-eastern part of Bangladesh (Figure 4.1).

Sample size

In the present study a total of 338 febrile patients were enrolled from the Matiranga Upazila Health Complex (UHC) from July 2010-May 2012.

Sample collection

Five ml of blood was taken from an adult subject and in case of children or minor subjects three ml of blood was obtained through veni-puncture. Two drops of sample were used for preparing thick and thin smear slides, one drop was used for Paracheck RDT, and the remaining samples were preserved in an EDTA tube and stored at -20°C.

Microscopy

The blood film was stained with Giemsa in phosphate buffer saline and examined under the compound microscope at a magnification of x1,000 for malaria parasites. Blood films were defined as negative if no parasite was observed in 100x oil immersion fields (magnification, x1,000) on thin film (Warhurst and Williams 1996). Declaring a slide positive or negative and initial speciation was routinely based on the examination of 200 fields in the Giemsa-stained thick film. A slide was considered positive when at least one parasite was found. After finding the first parasite, another 200 fields were completed for any mixed infection. If no parasite was found in 200 oil fields, the slide was considered negative. Density of the parasite was measured from thick blood smears by counting the number of parasites per 200 leukocytes and expressed as parasites/µl. In the case of 10 or less parasites, 500 leukocytes were counted.

Rapid diagnostic test

All samples were also tested by a RDT Paracheck (Orchid Biomedical System, India). Paracheck used *Plasmodium falciparum*-specific HRP-2 antigen and only can detect *P. falciparum* in the blood sample. RDT was used following the manufacturers instruction.

DNA extraction

DNA was extracted from 200 µl EDTA preserved blood samples using the QiaAmp blood mini kit (QIAGEN, Inc., Germany) following the manufacturer's instructions at the Parasitology Laboratory of icddr,b. DNA sample was stored at 4 °C until PCR could be completed. Briefly the procedure was as follow:

1. 20 µl of Qiagen Protease or Proteinase K was pipetted into the bottom of a 1.5ml micro-centrifuge tube containing 200µl blood. When the sample was less than 200µl, appropriate volume of Phosphate Buffer Solution (PBS) was added.

2. 200 µl of Lysis Buffer (Buffer AL) was added to the sample and was mixed by pulse vortex for 15 seconds.

3. The mixture was then incubated at 56ºC for 10 minutes.

4. After 10 minutes of incubation, the 1.5ml micro-centrifuge tube was centrifuged briefly to remove the droplets from inside the lid.

5. 200µl ethanol (96-100%) was added to the sample and mixed by pulse vortex for about 15 seconds. After mixing, the micro-centrifuge tube was briefly centrifuged to remove the drops from the inside of the lid.

6. 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.

7. 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.

8. 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.

9. 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.

10. 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.

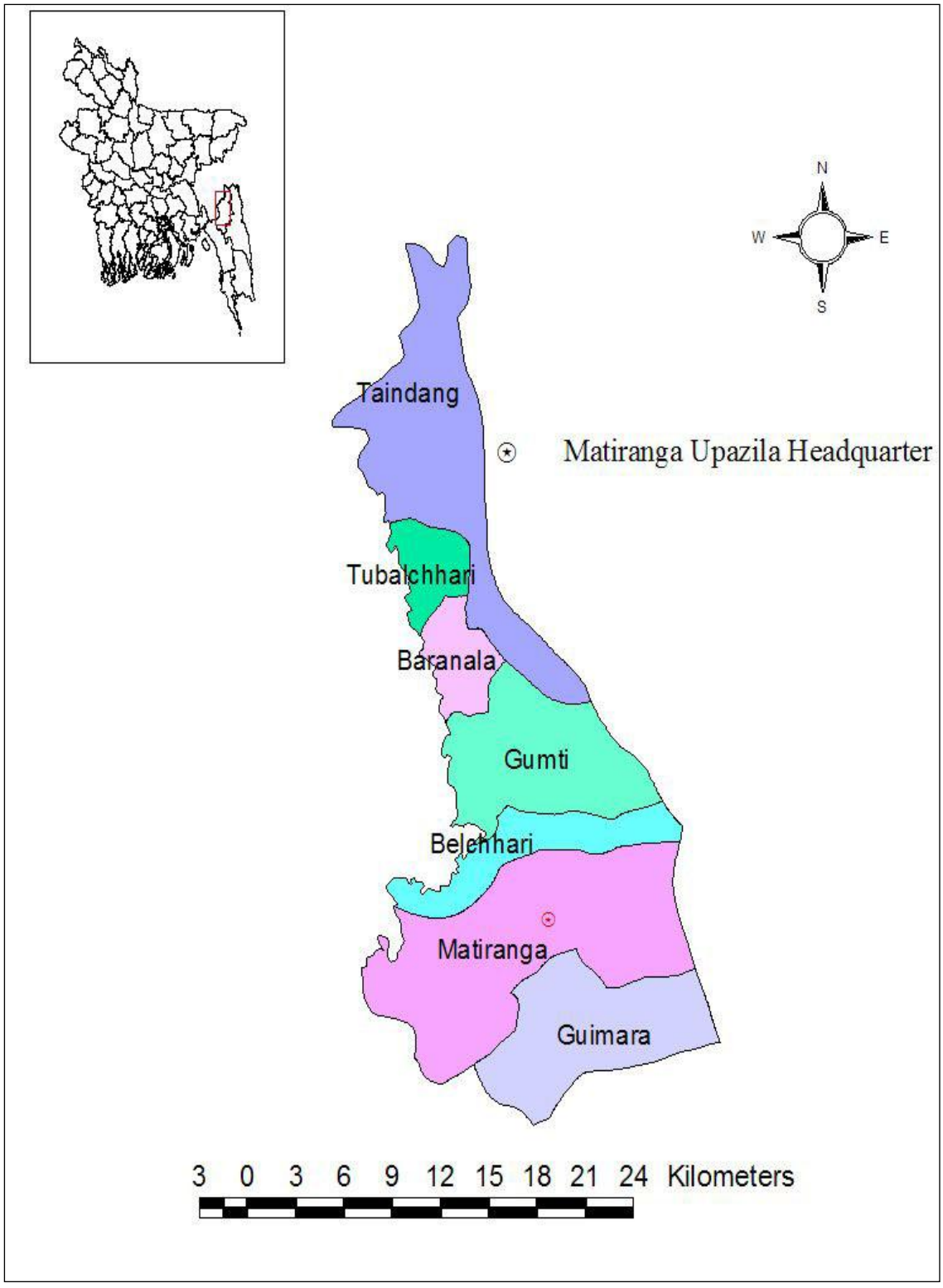


Figure 5.1: Map of Matiranga upazila up to union level.

Real-time PCR

Real-time PCR was done by the primer sets described by Perandin *et al.* (2004) with some modification to a single-plex reaction and instead of TaqMan probe, SYBR Green I dye was used for visualizing the amplification. PCR condition was also modified slightly to fit with Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen Corporation, USA) following the instructions of the manufacturer. Purified DNA templates were amplified in a BioRad CFX-96 real time system (BioRad, USA) with a species-specific primer set.

Primers:

FAL-F: 5'-CTT TTG AGA GGT TTT GTT ACT TTG AGT AA-3'

FAL-R: 5'-TAT TCC ATG CTG TAG TAT TCA AAC ACA A-3'

PCR Program:

1.	50ºC	2 min
2.	95ºC	2 min
3.	95ºC	1 min
4.	58ºC	1 min
5.	72ºC	1 min 30 sec
6.	Go to step 3	39x (for *P. falciparum*) 34x (for *P. vivax*)

The plate reading was taken after the extension at 72°C. C(t) for *P. falciparum* 38 cycle and C(t) for *P. vivax* 34 cycle. The melt curve was prepared from 50°C to 95°C with an increment of 0.5°C each after five seconds.

Figure 5.2: BioRad CFX-96 real time system (BioRad, USA) used in the present study.

Reaction Mixture:	**25 µL**
2X SYBR Green Super Mix	12.5 µL
50mM $MgCl_2$	1.5 µL
Forward Primer	0.8 µL (final concentration 320 nmol)
Reverse Primer	0.8 µL (final concentration 320 nmol)
dH_20	8.4 µL
Template DNA	1.0 µL

Use up to 3 µL of template DNA (from abdomen extraction eluted in 50 µL dH_20).

Briefly, a 25-µl PCR mixture was prepared using 1 µl of template DNA, 12.5 µl Platinum SYBR Green qPCR supermix (PlatinumR *Taq* DNA polymerase, SYBR Green I dye, Tris-HCl, KCl, 6 mM MgCl2, 400 µM dGTP, 400 µM dATP, 400 µM dCTP, 800 µM dUTP, uracil DNA glycosylase, and stabilizers), 320 nM concentration of each of parasite species-specific primer set. Amplification and detection were performed as follows: 50 °C for 2 min and 95 °C for 2 min. After that

95 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min 30 sec for a single cycle were performed. 40 cycles were considered for *P. falciparum*.

The plate reading was taken after the extension at 72 °C. The melt curve was prepared from 50 °C to 95 °C with an increment of 0.5°C each after five seconds.

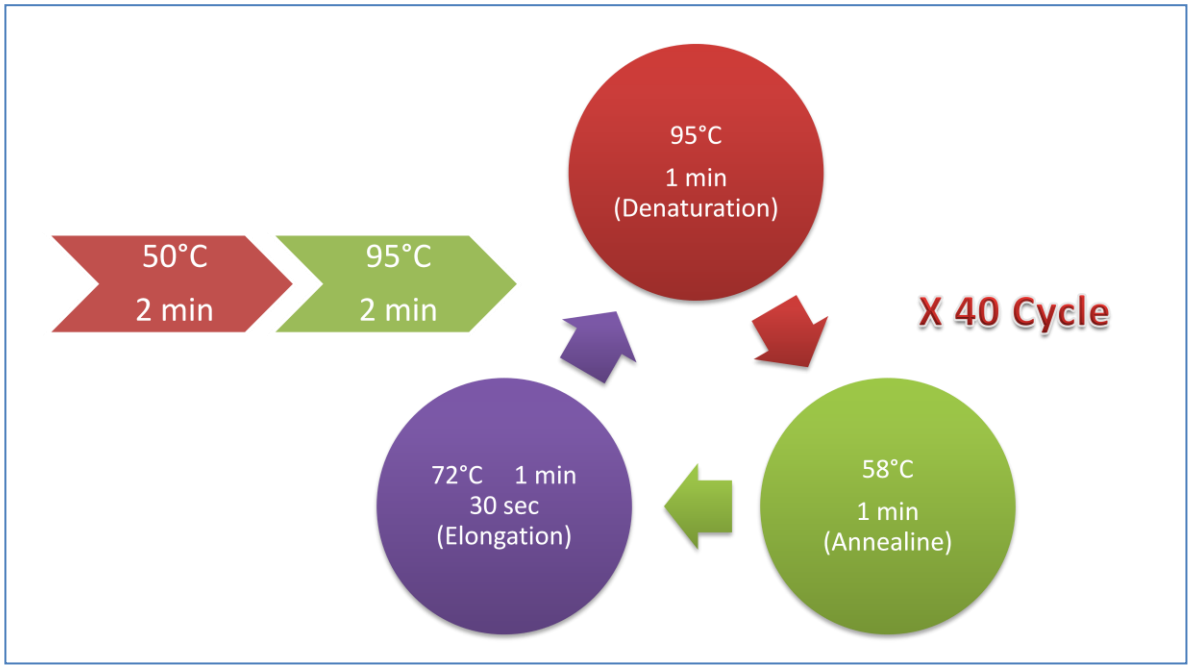


Figure 5.3: Steps in real-time PCR applied in the present study.

To establish the minimum number of parasites detectable by the *Plasmodium* SYBR Green assay (detection limit), blood samples from two patients infected, respectively, with *P. falciparum* (one patient) and *P. vivax* (one patient) were collected, and parasitaemia was calculated by using 200 WBC count as reference. The infected blood samples were diluted with uninfected erythrocytes from healthy individuals with known baseline erythrocyte counts. Ten-fold serial dilution was made to obtain a final parasitaemia of 1% (1 parasite/µl of blood) for each sample. All DNA aliquots purified from the dilutions were duplicated for real-time PCR assay. To estimate the analytical specificity of the *Plasmodium* real-time PCR assay, DNA from *in vitro* culture samples of other protozoan parasites, such as *Entamoeba histolytica* and *Leishmania donovani* were used. The clinical sensitivity and specificity of the modified *Plasmodium* real-time PCR assay for detecting and identifying malaria

parasites were calculated on 338 whole-blood samples, microscopy as the gold standard and vice- versa.

Analysis of data

The performance of each method was calculated by means of sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) using microscopy and modified real-time PCR as gold standard. SPSS software version 11.5 (SPSS Inc., USA) was used for calculating the kappa coefficient (k) of the tests for each association using the X^2 test. Sensitivity, specificity, positive predictive value and negative predictive value were calculated using the 'diagt' command of the STATA software version 10 (Stata Corp, USA) (Seed 2001).

Definitions and formula

Following terminologies have been used to compare our new test with to a reference test (here microscopy).

1. Clinical Sensitivity: The percentage of persons who are truly positive that test positive by our new test. Formula for Sensitivity is given below:

$$\text{sensitivity} = \frac{\text{number of true positives}}{\text{number of true positives} + \text{number of false negatives}}$$

$$= \frac{\text{number of true positives}}{\text{total number of sick individuals in population}}$$

$$= \text{probability of a positive test, given that the patient is ill}$$

2. Clinical Specificity: The percentages of persons are truly negative that are positive by our new test. Formula for Specificity is given below:

$$\text{specificity} = \frac{\text{number of true negatives}}{\text{number of true negatives} + \text{number of false positives}}$$

$$= \frac{\text{number of true negatives}}{\text{total number of well individuals in population}}$$

$$= \text{probability of a negative test given that the patient is well}$$

3. Positive Predictive Value (PPV): Percentage of persons with a positive test who are truly positive. Formula for PPV is given below:

$$PPV = \frac{sensitivity \times prevalence}{sensitivity \times prevalence + (1 - specificity) \times (1 - prevalence)}$$

4. Negative Predictive Value (NPV) = Percentage of persons with a negative test that are truly negative (eg. uninfected). Formula for NPV is given below:

$$NPV = \frac{specificity \times (1 - prevalence)}{(1 - sensitivity) \times prevalence + specificity \times (1 - prevalence)}$$

Kappa:(κ) : chance-corrected agreement, i.e. statistic used to evaluate strength of agreement over that expected by chance, such that the close1 to "l" that the kappa value is, the more perfect the agreement between the tests is, beyond that which would be expected by chance (1= perfect agreement, 0.4 - 0.6= moderate agreement, 0 -0.2= poor to no agreement). Formula for Kappa is given below:

$$\mathbf{kappa} = \frac{(p_o - p_c)}{(1 - p_c)}$$

where p_o is the observed proportion of agreement
and p_c is the proportion expected by chance.

Melting curve analysis

Melting curve analysis is an assessment of the dissociation-characteristics of double-stranded DNA during heating. As the temperature is raised, the double strand begins to dissociate leading to a rise in the absorbance intensity, hyperchromocity. The temperature at which 50% of DNA is denatured is known as the melting point, though it is an inaccurate term as it has very little to do with a traditional melting point.

Results and Observations

Microscopy

Malaria parasite *Plasmodium falciparum* was detected in 171 (50.4%) patients by microscopy. Of them three (1.6%) patients had a mixed infection (Table 4.1). Overall, high parasite count was observed in microscopy. Parasite count ranged from 16 to 261,480 parasites/µl of blood. A median number of parasite counts of 19,960 [interquartile range (IQR) 6,280-48,320] parasites/µl of blood was found in 171 *P. falciparum* positive patients. Only six (3.5%) of the samples were below 100 parasites/ µl whereas, 118 (69%) had a count of more than 10,000 parasites/ µl of blood.

Rapid diagnostic test

As like microscopy Paracheck, rapid diagnostic test gave 171 *P. falciparum* positive results (50.4%). However, as microscopy positive samples were not having the same results for Pracheck RDT.

Analytical sensitivity Real-Time PCR

In the present investigation, after having the successful amplification by real-time PCR method detection limit (analytical sensitivity) was established following the typical amplification curve. Typical displays (amplification plots) for *P. falciparum* by the SYBR Green I PCR assay provided by Bio Rad CFX-96 are shown in Figures 5.4. Positive signals by means of cycle threshold [CT] value were obtained for all dilutions, with a detection limit of 5-10 parasites/µl for *P. falciparum* in different experiments. The new real-time PCR could detect less than 1 parasite/uL from clinical sample.

Reproducible linearity of over a 10,000-fold range was shown by CT values. A significant correlation coefficient was found for the mean CT values and parasitaemia (*P. falciparum,* R^2=0.982) (Figure 5.5). For non-*Plasmodium* protozoan DNA (*E. histolytica* and *L. donovani*) and blood DNA samples of healthy human subjects no signal was obtained by the SYBR Green real-time PCR.

Melt curve analysis

The melting peak for *P. falciparum* was found at 74.5 °C from the corresponding positive controls respectively (Figure 5.6). Any amplification other than these two melting temperatures was excluded as false amplification.

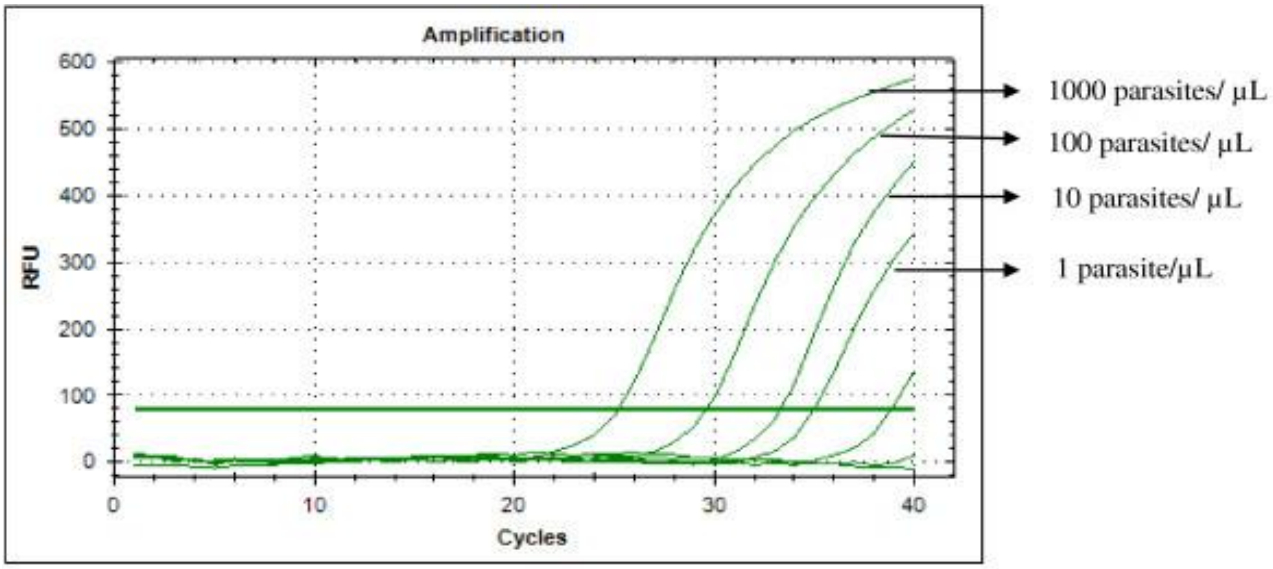


Figure 5.4: Typical amplification curve for *P. falciparum*.

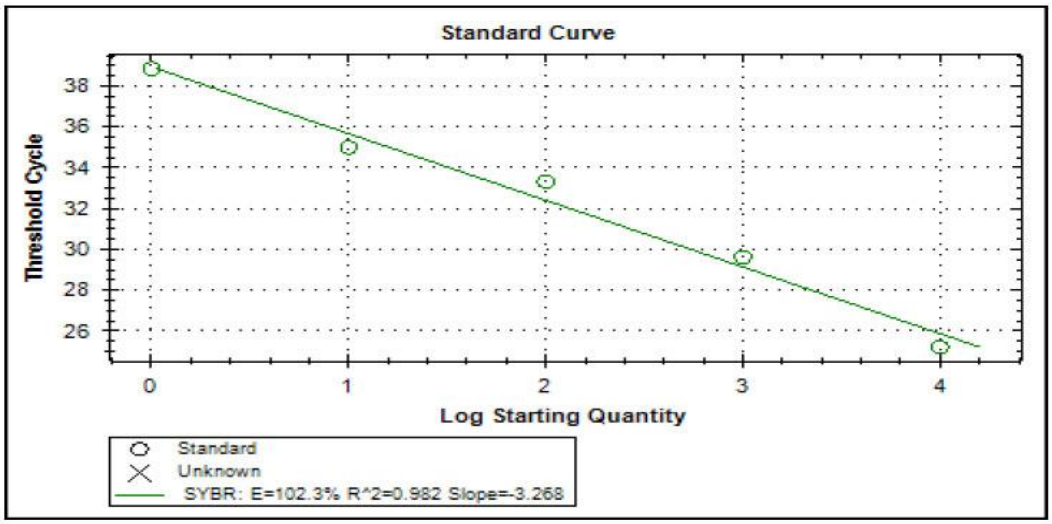


Figure 5.5: Standard curve for *P. falciparum* produced against CT values and logarithm of parasite count/µL of blood (R^2=0.982).

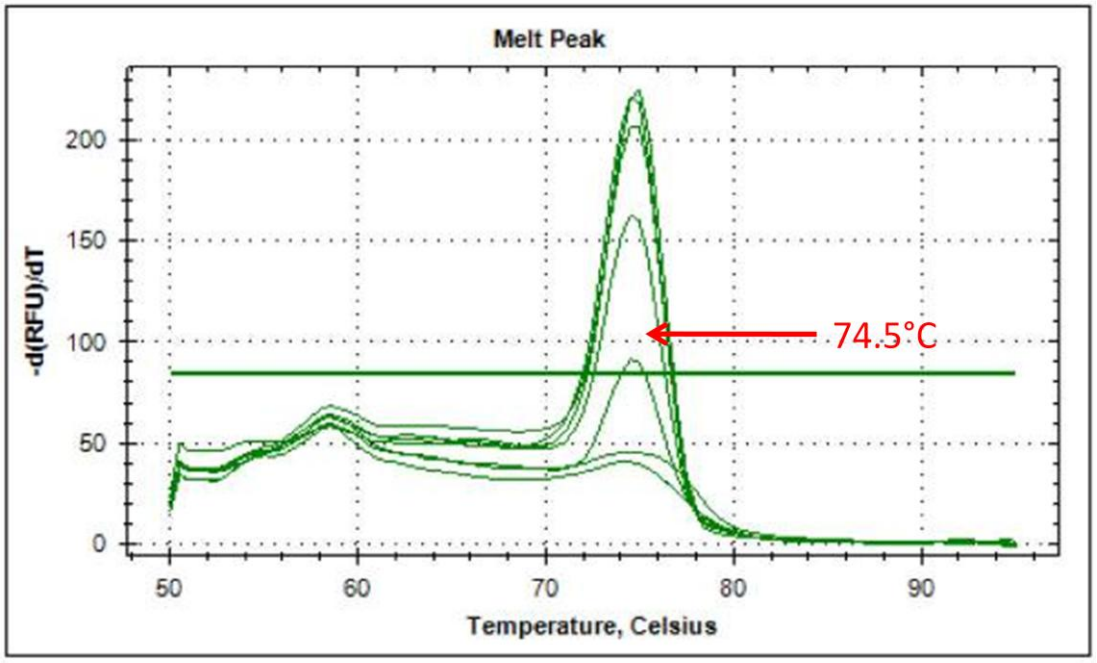


Figure 5.6: Typical melt curve of *P. falciparum* showing peak at 74.5 ˚C.

Table 5.1: *P. falciparum* diagnosis Microscopy, RDT vs. Real-Time PCR (N=338).

Test	***P. falciparum* Positive (%)**	**Negative (%)**	**Total**
Microscopy	171(50.6)	167 (49.4)	338
Parachech (RDT)	171(50.6)	167 (49.4)	338
Real-time PCR	170 (50.3)	168 (49.7)	338

Evaluation of Real-Time PCR

Using the real-time PCR assay results 170 (50.3%) samples were found positive for any malarial infection (Table 5.1). Sensitivity, specificity, positive predictive value, negative predictive value, and kappa (k) of PCR assay compared to microscopy are given in table 5.2. For the detection of *P. falciparum* (including mixed infection), modified real-time PCR assay had 97.1% (95% CI: 93.3-99) sensitivity and 97.6% (95% CI: 94-99.3) specificity respectively (Table 5.2).

Real-time PCR vs. Parasite count

According to parasite count (per uL) by microscopy the newly established real-time PCR provided good result. Based on parasite count 7 categories were made: 1-100/ uL, 101-200/ uL, 201-500/ uL, 501-1,000/ uL, 1,001-5,000/ uL, 5001-10,000/ uL and >10,000/ uL. Among the positive samples 118 were in the >10,000/ uL category and real-time PCR could peal all of those, thus success rate was 100% (Table 5.3). This success rate was same for all categories except 101-200 group of which real-time PCR could peak only 3 out of 7 samples as positive. Probable reason for this result has been explained in the discussion section.

Table 5.2: Sensitivity, specificity, positive predictive value, and negative predictive value of real-time PCR versus microscopy.

Sensitivity (95% CI)	**Specificity (95% CI)**	**PPV* (95% CI)**	**NPV** (95% CI)**	**Kappa (κ)**
97.1 (93.3-99.0)	97.6 (94.0-99.3)	97.6 (94.1-99.4)	97.0 (93.2-99.0)	0.95

* PPV = positive predictive value

** NPV= negative predictive value

Table 5.3: Detection of malaria parasites according to microscopic count by real time-PCR.

Category	Positive (N)	Positive (%)	Negative (N)	Negative (%)	Total
1-100 uL	6	100	0	0	6
101-200 uL	3	42.9	4	57.1	7
201-500 uL	2	100	0	0	2
501-1,000 uL	7	100	0	0	7
1,001-5,000 uL	17	100	0	0	17
5,001-10,000 uL	13	100	0	0	13
> 10,000 uL	118	100	0	0	118
Negative	4	2.4	164	97.6	168
Total	170	100	168	100	338

Sensitivity according to parasite count

Sensitivity of the newly developed real-time PCR method has been calculated according to different parasite count. Except for the count group 101-200 uL which had 42.9% sensitivity rate (95% CI, 11.8-%-78.8%), all six categories were 100% sensitive (95% CI 19.8%-100%) (Table 5.4). Due to small number of samples (N=5 to 17) in five categories 1-100/ uL, 201-500/ uL, 501-1,000/ uL, 1,001-5,000/ uL and 5001-10,000/ uL the lower limit was within 78%. Finally a sensitivity curve of real-time PCR was produced against different parasite count category (Figure 5.7).

Table 5.4: Sensitivity, specificity, positive predictive value, and negative predictive value of real-time PCR according to parasite count.

Category	Sensitivity	95% Confidence Interval	
		Lower limit	Upper limit
1-100 uL	100%	51.7%	100%
101-200 uL	42.9%	11.8%	78.8%
201-500 uL	100%	19.8%	100%
501-1,000 uL	100%	56.1%	100%
1,001-5,000 uL	100%	77.1%	100%
5,001-10,000 uL	100%	71.7%	100%
> 10,000 uL	100%	96.1%	100%
Total	97.1%	93.3%	99.0%

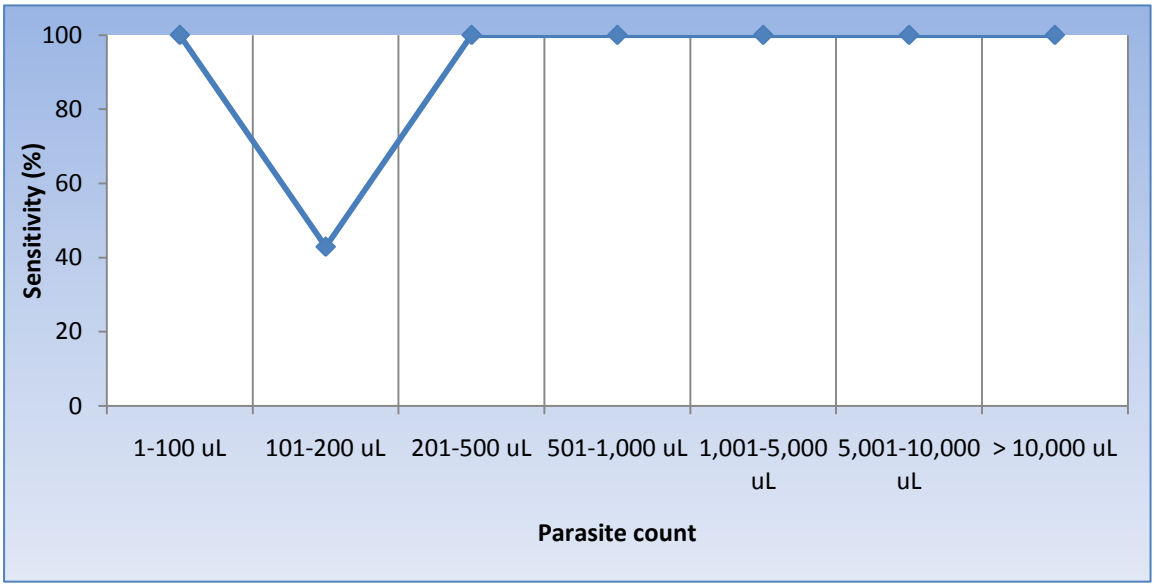


Figure 5.7: Sensitivity of real-time PCR according to parasite count categories.

Discussion

Although in Bangladesh, *Plasmodium falciparum* and *P. vivax* are the two common prevalent parasites, the majority of malaria cases are caused by *P. falciparum* (M&PDC 2010). However, their ratio varies from time to time. During the nationwide malaria prevalence survey in 2007 based on Falcivax RDT, 90 % *P. falciparum* infection was found and the remaining 10% infection was due to pure *P. vivax* or mixed infection (Haque *et al.* 2009a).

Analytical sensitivity Real-Time PCR

The newly developed real-time PCR could detect less than 1 parasite/uL from clinical sample. This rate is similar to previously described real-time PCR method by Perandin *et al.* (2004), Shokoples (2009) and Tran *et al.* (2014). Lee *et al.* (2002) established a real-time fluorescence PCR method with specific primers and a labeled probe which had a sensitivity of 0.002 pg of *P. falciparum* DNA, equivalent to 0.1 parasite. Their method was also specific for all *P. falciparum*, *P. malariae*, *P. ovale*, and *P. vivax* parasites. However, the present study demonstrated the establishment of a real-time PCR assay modified with SYBR Green dye for the detection of *P. falciparum* as an alternative. Probe/TaqMan-based real-time PCR is expensive and most of the reference lab in middle and low income country cannot afford. Instead of probe, SYBR Green can be an affordable alternative.

Melting curve analysis

During melt-curve analysis the peak for *P. falciparum* was found at 74.5 °C from the corresponding positive controls respectively (Figure 5.6). Melt curve is specific to PCR target site and may vary within the same species if the target site changed. Our melt-curve analysis reveals the similar pattern as described by Taylor *et al.* 2011. According to Mangold *et al.* 2005 real-time PCR with melting curve analysis could be a rapid and objective supplement to the examination of Giemsa-stained blood smears and may replace microscopy following further validation.

Evaluation of real-time PCR

For the detection of *P. falciparum*, the newly developed real-time PCR assay had 97.1% sensitivity and 97.6% specificity. Throughout the world, a different rate has been observed for establishment of a new molecular method. Real-time PCR for malaria parasites may vary from 90% (Kamau *et al*. 2011), 94.1% (Mangold *et al.* 2005) to 100% (Perandin *et al.* 2004).

Newly developed real-time PCR provided 97.6 % specificity (95% CI: 94.0%-99.3%) which is also a comparable result globally. Specificity may also vary from 91.2% (Rantala *et al*. 2010) to 100% (Perandin *et al.* 2004).

Sensitivity according to parasite count

Except for the count group 101-200 uL, which had 42.9% sensitivity rate (95% CI, 11.8-%-78.8%) all six categories were found to have 100% sensitive (95% CI 19.8%-100%) (Table 5.3 and Figure 5.7).

In the present study, the newly developed real-time PCR method missed four samples which were positive as *P. falciparum* in microscopy and picked 4 samples as positive for *P. falciparum* which were negative in microscopy. The reason could be the limitation of microscopy as the low parasite counted sample could easily be misdiagnosed as *P. falciparum* where sample originally may be *P. vivax* (Perandin *et al.* 2004 and Cnops *et al*. 2010).

In the present study, more than 50% of the febrile patients with suspected malaria had a positive blood slide, indicating that over half of the suspected cases referred to this hospital (Matiranga UHC) had malaria. A high percentage of malaria cases among the febrile cases of this area could be due to a high prevalence of asymptomatic malaria cases at the community (Haque *et al*. 2009 b) .

CHAPTER 6

ESTABLISHMENT AND IMPROVEMENT OF LOOP MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) FOR *PLASMODIUM FALCIPARUM* DIAGNOSIS

Materials and methods

Loop mediated isothermal amplification (LAMP)

LAMP is a single tube technique for the amplification of DNA (Notomi *et al.* 2000). This method may be useful in future as a low cost alternative to detect certain diseases. It may be combined with a reverse transcription step to allow the detection of RNA.

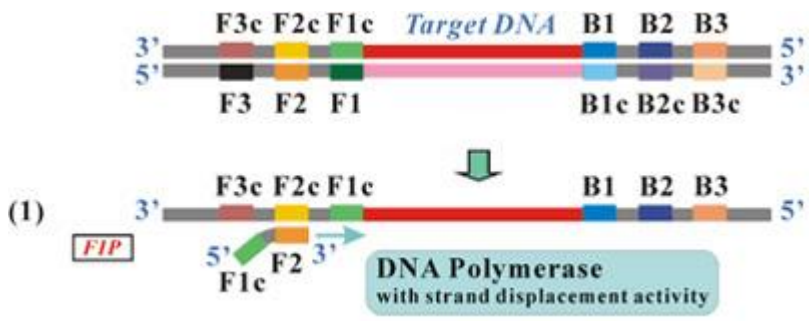
Technique

LAMP is isothermal nucleic acid amplification. In contrast to the polymerase chain reaction (PCR) technology in which the reaction is carried out with a series of alternating temperature steps or cycles, isothermal amplification is carried out at a constant temperature, and does not require a thermal cycler.

When the target gene (DNA template as example) and the reagents are incubated at a constant temperature between 60-65°C, the following reaction steps proceed:

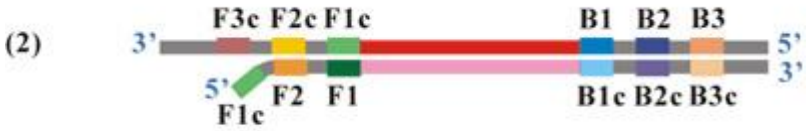
Step 1

As double stranded DNA is in the condition of dynamic equilibrium at the temperature around 65°C, one of the LAMP primers can anneal to the complimentary sequence of double stranded target DNA, then initiates DNA synthesis using the DNA polymerase with strand displacement activity, displacing and releasing a single stranded DNA. With the LAMP method, unlike with PCR, there is no need for heat denaturation of the double stranded DNA into a single strand. The following amplification mechanism explains itself when the FIP (forward inner primer) anneals to such released single stranded template DNA.



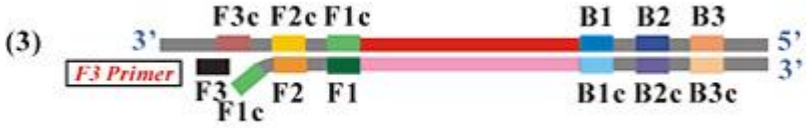
Step2

Through the activity of DNA polymerase with strand displacement activity, a DNA strand complementary to the template DNA is synthesized, starting from the 3' end of the F2 region of the FIP.



Step3

The F3 Primer anneals to the F3c region, outside of FIP, on the target DNA and initiates strand displacement DNA synthesis, releasing the FIP-linked complementary strand.



Step4

A double strand is formed from the DNA strand synthesized from the F3 Primer and the template DNA strand.

Step5

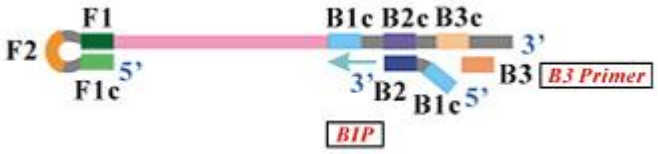
The FIP-linked complementary strand is released as a single strand because of the displacement by the DNA strand synthesized from the F3 Primer. Then, this released single strand forms a stem-loop structure at the 5' end because of the complementary F1c and F1 regions.

STEP 6

The single strand DNA in Step (5) serves as a template for BIP (backward inner primer)-initiated DNA synthesis and subsequent B3-primed strand displacement DNA synthesis. The BIP anneals to the DNA strand produced in Step (5). Starting from the 3' end of the BIP, synthesis of complementary DNA takes place. Through this process, the DNA reverts from a loop structure into a linear structure. The B3 Primer anneals to the outside of the BIP and then, through the activity of the DNA polymerase and starting at the 3' end, the DNA synthesized from the BIP is displaced and released as a single strand before DNA synthesis

from the B3 Primer.

(6)



STEP7

Double stranded DNA is produced through the processes described in Step (6).

(7)

STEP 8

The BIP-linked complementary strand displaced in Step (6) forms a structure with stem-loops at each end, which looks like a dumbbell structure. This structure serves as the starting structure for the amplification cycle in the LAMP method (LAMP cycling). The above process can be understood as producing the starting structure for LAMP cycling.

(8)

Basic Principle (8)-(11) (Cycling Step)

A dumbbell-like DNA structure is quickly converted into a stem-loop DNA by self-primed DNA synthesis. FIP anneals to the single stranded region in the stem-loop DNA and primes strand displacement DNA synthesis, releasing the previously synthesized strand. This released single strand forms a stem-loop structure at the 3' end because of complementary B1c and B1 regions. Then, starting from the 3' end of the B1 region, DNA synthesis starts using self-structure as a template, and releases FIP-linked complementary strand (Step (9)). The released single strand then forms a dumbbell-like structure as both ends have complementary F1 - F1c and B1c - B1 regions, respectively (Step (11)). This structure is the 'turn over' structure of the structure formed in Step (8). Similar to the Steps from (8) to (11), structure in Step (11) leads to self-primed DNA synthesis starting from the 3' end of the B1 region. Furthermore, BIP anneals to the B2c region and primes strand displacement DNA synthesis, releasing the B1-primed DNA strand. Accordingly, similar structures to Steps (9) and (10) as well as the same structure as Step (8) are produced. With the structure produced in Step (10), the BIP anneals to the single strand B2c region, and DNA synthesis continues by displacing double stranded DNA sequence. As a result of

this process, various sized structures consisting of alternately inverted repeats of the target sequence on the same strand are formed.

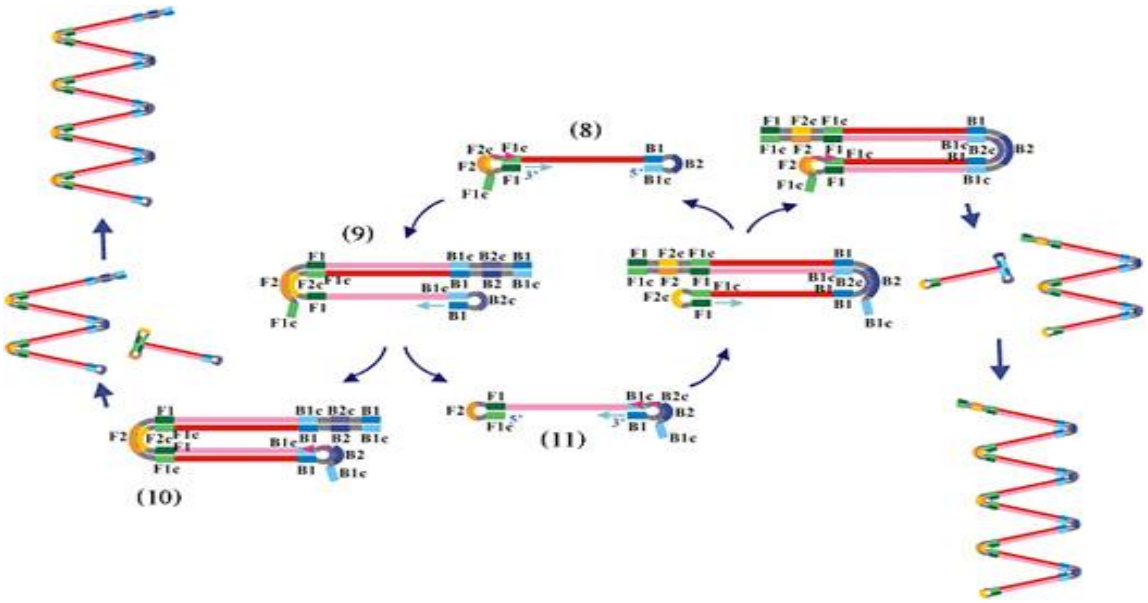


Figure 6.1: Cyclic stage of LAMP reaction.

Sampling source

Out of 338 samples obtained from the Matiranga Health Complex 106 microscope-positive *Plasmodium falciparum* mono-infection and 105 microscopically negative samples were included in the present study. Thus, a total of 211 samples were taken in to the LAMP experiment. Study area and sample collection procedures were described in previous section (chapter 5). In addition a negative panel consists of 43 DNA samples of *P. vivax*, *P. malariae. Leishmania donovani* and tuberculosis were also tested to check analytical specificity of the LAMP methods.

Microscopy

Thick and thin smear slides were prepared in duplicate by using two drops of blood for each sample. Microscopy was performed according to standard procedures (which have been described in the previous chapter).

DNA extraction

DNA was extracted from 200µL preserved whole blood using QiaAmp blood mini kit (Qiagen GmbH, Germany) following manufacturer's instructions. Detail process of DNA extraction has been described in previous chapter.

Real-time PCR

Real-time PCR was done on the extracted DNA according to the procedure described in the earlier section (chapter 5) using Invitrogen^{TM} SYBR Green I supermix UDG (Life Technologies Corporation, USA).

LAMP Methods

Initially LAMP method was carried out following the protocol and primer set described by Poon *et al.* (2006), although pre-addition of 120µM HNB was introduced in the reaction mixture. 2 µL of extracted DNA was used as template for amplification. The tubes were heated at 60˚C for 60 minutes in a heat block (Figure 6.2). Later LAMP experiment was performed with a new primer set designed for the present study. Poon *et al.* (2006) used following primer set in their experiment:

Table 6.1: List of old LAMP primers (Poon *et al.* 2006) used for this experiment.

Primer Name	**Sequence**
F1P (F1C+F2)	AGCTGGAATTACCGGCGGCTGGGTTCCTAGAAAACAATTGG
B1P (B1+B2C)	TGTTGCAGTTAAAACGTTCGTAGCCCAAACCAGTTTAAATG AAAC
F3	TGTAATTGGAATGATAGGAATTTA
B3C	GAAAACCTTATTTTGAACAAAGC
LPF	GCACCAGACTTGCCCT
LPB	TTGAATATTAAAGAA

Composition of master mix:

Reagent name	Amount/ concentration
Tris-HCl	40 mM
KCl	20mM
(NH4)2SO4	20mM
MgSO4	8mM
Tween 20	0.1%
Primer mix	9.5µL
HNB	120µM
Bst polymerase	1 µL (8U)
Total reaction volume	25µL

Assay procedure

A 2X reaction mixture was prepared comprising 40mM Tris-HCl (pH 8.8), 20mM $(NH_4)_2SO_4$ (pH 8.8), 20 mM KCl, 16mM $MgSO_4$, 1M betain, 2.4 mM each of the DNTPs, 120µM HNB and 0.01% Tween 20. Primer mixture was prepared with 2.4µM of F1P and B1P; 0.6µM of LPF and LPB; 0.2µM of F3C and B3 primers. All reactions were done in 25µL tubes using 2µL of template DNA, 12.5µL 2X reaction mixture, 9.5µL of primer mixture (Table 6.2) and 1µL (8U) of *Bst* polymerase. All tubes were heated at 60ºC for 40 minutes in a heat block. The changes in the color of the reaction mixture for both LAMP methods were visualized by the naked eyes as these became turbid (Figure 6.3).

Table 6.2: Composition of primer mixture.

Name	**Amount**
100 µM F1P	0.6 µL
100 µM B1P	0.6 µL
100 µM LPF	0.4 µL
100 µM LPB	0.4 µL
100 µM F3	0.2 µL
100 µM B3C	0.2 µL
H_2O	7.8 µL
Total	9.5µL

Figure: 6.2: Heat block (heating chamber) used for LAMP assay.

Primers for new malaria LAMP

A slightly different region in the 18S rRNA gene of *P. falciparum,* 3D7 type was selected for using NCBI-BLAST tool. Primers were designed with the Primer Explorer V4: an online primer designing tool (http://primerexplorer.jp/elamp4.0.0/index.html) developed by Eiken Chemical Co. LTD, Japan. Primer sequences are listed in Table 6.3.

For the newly designed primer set, a 2X reaction mixture was prepared comprising 40mM Tris-HCl (pH 8.8), 20mM $(NH_4)_2SO_4$ (pH 8.8), 20 mM KCl, 16mM $MgSO_4$, 1M betain, 2.4 mM each of the DNTPs, 120µM HNB and 0.01% Tween 20. Primer mixture was prepared with 2.4µM of F1P and B1P; 0.6µM of LPF and LPB; 0.2µM of F3C and B3 primers. All reactions were done in 25µL tubes using 2µL of template DNA, 12.5µL 2X reaction mixture, 9.5µL of primer mixture and 1µL (8U) of *Bst* polymerase. All tubes were heated at 60ºC for 40 minutes in a heat block.

Table 6.3: List of primers designed for new malaria LAMP.

Primer Name	Sequence
Pf -F3	TGATAGGAATTTACAAGGTTCC
Pf -B3	GAAAACCTTATTTTGAACAAAGC
Pf-F1P	TGCTATTGGAGCTGGAATTACCGTAGAGAAACAATTGGAGGGC
Pf-B1P	GTTGCAGTTAAAACGTTCGTAGTTGTGGTTTTCCCAAACCAGTT
Pf-LPF	GCTGCTGGCACCAGACTT
Pf-LPB	ATTAAAGAATCCGATGTTTCATTT

The changes in the color of the reaction mixture for both LAMP methods were visualized by the naked eye. The tubes containing detectable amount of *P. falciparum* DNA turned to sky blue from the initial violet color (Fig. 6.4).

In order to set the reaction time, LAMP was carried on serially diluted DNA samples with parasitemia of 10000, 1000, 100, 10, 5 and 1 initially in the present study. Each sample was amplified for five rounds. In each round of assay, reactions with five parasites/µL DNA as template was amplified and changed the color within 30-32 minutes. Sometimes reactions with one parasite/ µL DNA as template was amplified within around 35-36 minutes (Figure 6.3). Incubation for longer time didn't show any color change. Therefore, 40 minutes has been chosen as reaction time.

Analytical sensitivity and specificity

Two confirmed microscopically-positive *P. falciparum* samples were selected and slides were analyzed by three independent microscopes. Parasitemia were calculated according to WHO standard procedures. Average parasite count was made using three independent results from each microscope for each of the samples. These samples were diluted with a healthy negative control blood sample to establish the parasitemia of 10000, 1000, 100, 10, 5 and 1 parasite/µL. Then DNA was extracted and LAMP reactions were carried out on DNA of the diluted samples for five rounds following the procedures described above (Figure 6.4).

A negative control panel was comprised of 20 *P. vivax*, 3 *P. malariae*, 10 *Leishmania donovani*, 10 *Mycobacterium tuberculosis* positive blood samples and DNA was extracted. Infection status of the samples was confirmed by PCR. Then LAMP reaction was carried out to assess the cross-reactivity of the newly designed primers with this set of nonmalaria microbial DNA in blood.

Data analysis

All data analyses were conducted in STATA version 11.0 (StataCorp, College Station Texas, USA). Sensitivity, specificity, predictive values and accuracy were calculated with their corresponding 95% confidence intervals (95% CI) by the McNemar test and exact the McNemar test using the 'diagt' command.

Definitions and formula

Definitions and formulas for sensitivity, specificity, PPV, NPV and Kappa were provided in the previous section (Chapter 5).

Results and Observations

Limit of detection of old vs. new LAMP

The old LAMP primers by Poon *et al.* (2006) detected a minimum of100 parasites/µL within 60 minutes. This experiment was repeated five times with same end results. No alteration in the initial color was observed after heating the samples up to 90 minutes. Conversely, LAMP with newly designed primers successfully detected 5 parasites/µL within 35 minutes. This experiment was repeated five times with same end results. Out of the five repeated experiments, additional heating to 60 min resulted detection of one parasite/µL in two repeated amplifications. Typical LAMP reaction in Old and new methods are given Figure 6.3 and 6.4. An image of detection limit of new LAMP is provided in Figure 6.5.

All the 43 samples in negative panel of *P. vivax, P. malariae, Leishmania donovani* and tuberculosis tested negative by both of the LAMP primer sets. Thus analytical specificity with this panel was 100% for both primer sets.

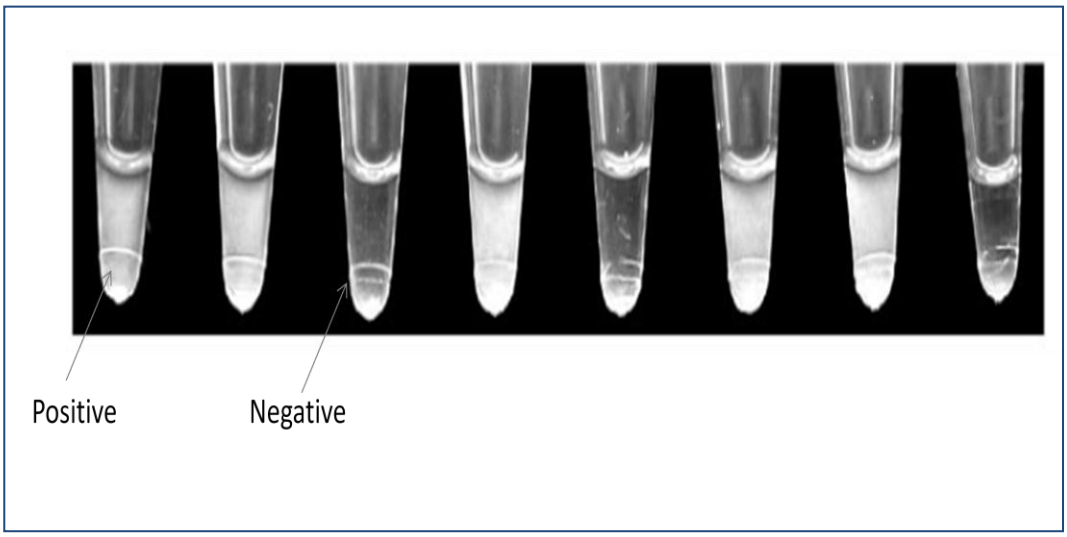


Figure 6.3: Typical LAMP reaction in the old method.

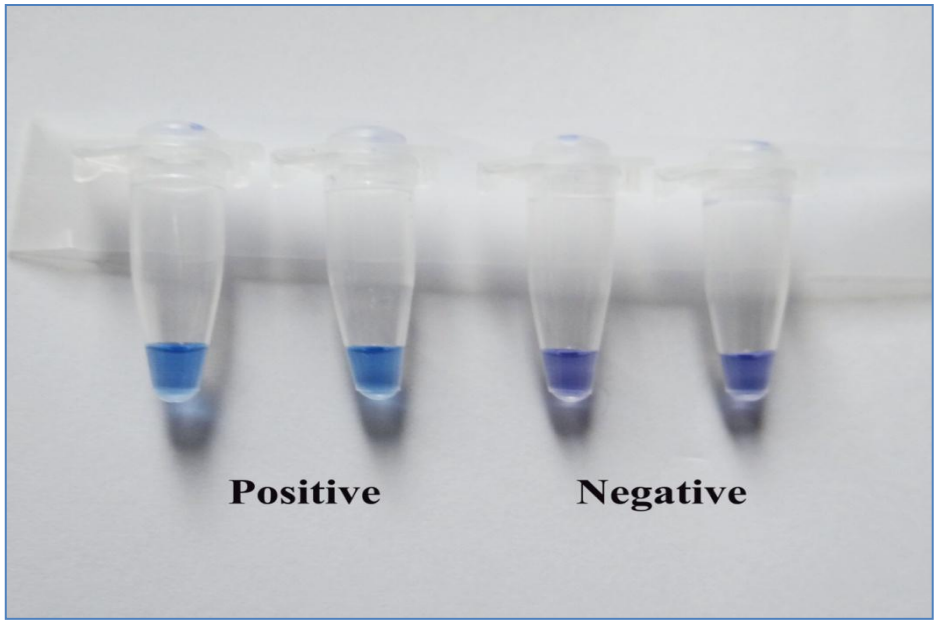


Figure 6.4: Typical LAMP reaction in the new method.

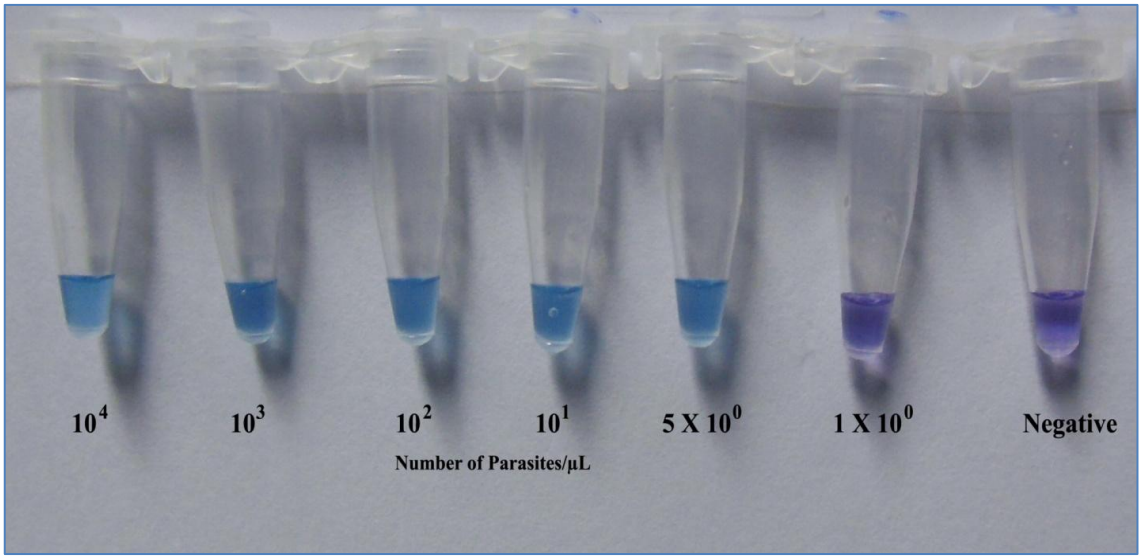


Figure 6.5: Analytical detection of *P. falciparum* DNA by the new LAMP method.

Detection against Microscopy and real-time PCR

Out of 211 DNA samples, 106 were positive in microscopy and 108 by real-time PCR. Out of 106 microscopy-positive samples old LAMP could detect 102 whereas, new LAMP could detect 105. On the other hand, out of 108 real-time PCR positive samples old LAMP could detect 103 but the new LAMP detected all 106. Out of 105 negative samples 2 samples were gave positive response in old LAMP but only 1 by the new LAMP method (Table 6.4).

Sensitivity and specificity

Old LAMP method was 96.2% (95% CI, 90.06-99.0) sensitive and 98.1% (95% CI, 93.3-99.8) specific in comparison with microscopy. Meanwhile, sensitivity decreased slightly to 95.4% (95% CI, 88.4-98.0) with somewhat improved specificity when real-time PCR was considered as the reference method (Table 6.5). LAMP with newly designed primers was 99.1% (95%CI, 94.9-100) and 98.1% (95CI%, 93.5-98.8) sensitive compared to microscopy and real-time PCR respectively while specificity was 99% (95% CI, 94.8-100) and 100% (95% CI, 96.5-100) for the assessment of *P. falciparum* DNA samples (Table 6.5). Positive predictive value (PPV), negative predictive values (NPV), agreement of the both LAMP methods in comparison with microscopy and real-time PCR are presented in Table 6.5.

Table 6.4: Comparative detection of LAMP methods with Microscopy and real-time PCR as reference methods.

Test	Result	Microscopy		Real Time PCR	
		Positive	Negative	Positive	Negative
Old LAMP	Positive	102	2	103	1
	Negative	4	103	5	102
New LAMP	Positive	105	1	106	0
	Negative	1	104	2	103

Table 6.5: Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of LAMP methods in comparison with microscopy and real-time PCR.

Reference method	**Method**	**Sensitivity (95% CI)**	**Specificity (95% CI)**	**PPV (95% CI)**	**NPV (95% CI)**	**Kappa κ**
Microscopy	Old LAMP	96.2% (90.6-99.0)	98.1% (93.3-99.8)	98.1% (93.2-99.8)	96.3% (90.7-99.0)	0.94
	New LAMP	99.1% (94.9-100.0)	99.0 % (94.8-100)	99.1% (94.9-100)	99.0% (94.8-100)	0.98
Real-Time PCR	Old LAMP	95.4% (89.5- 98.5)	99.0% (94.7-100)	99.0% (94.8-100)	95.3% (89.4-98.5)	0.94
	New LAMP	98.1% (93.5 -99.8)	100.0% (96.5-100)	100.0% (96.6-100)	98.1% (93.3-99.8)	0.98

Discussion

LAMP method comprising auto cycling and strand displacement DNA synthesis has become superior to other nucleic acid amplification techniques due to its simplicity, rapidity, sensitivity, specificity along with cost effectiveness (Abdul-Ghani *et al.* 2012). The LAMP method is very robust and rare mutations do not affect its efficacy unless they occur in the extreme ends of the primer binding sites (Notomi *et al*. 2000).

Parasite detection limit of old vs. new LAMP

The LAMP primer sets assessed in the present study were able to detect clinical *falciparum* malaria with great accuracy in comparison with microscopy and real-time PCR. LAMP with newly designed primers is more sensitive as it can detect a minimum of five *P. falciparum* parasites/µL of human blood. But with the old primer set Poon *et al.* (2006) the detection limit was obtained 100 copies from clinical samples. The newly designed primer set has overlapping regions with the primer set described by Poon *et al.* (2006) in the F3 region which is shown in gray in (Figure 6.6). This primer set can efficiently bind with three copies 18S rRNA genes residual of at least three different chromosomes of the same parasite (NCBI BLAST request ID: DD77A3ZC0R, DD7KBG8J016, DD80J0X6013).

As the newly developed primer set can detect 5 parasites/µL, it required approximately 15 copies of template to obtain significant visible amplification. However, Notomi *et al.* (2000), in their initial design mentioned that LAMP method can conduct visible amplification from six copies of template within 45 minutes when primer designing and reaction conditions are perfectly optimized. The newly developed primer set have failed to detect two of the real-time PCR positive samples where one of them was microscopically positive with a low parasitemia (16 parasites/µL). These real-time PCR-positive samples had high threshold values and low RFU. Both the samples also tested negative by Poon LAMP. Despite this minor incongruity in sensitivity, the newly developed primer set was 100% successful in avoiding false positive diagnosis due to cross-reactivity.

Detection against Microscopy and real-time PCR

Due to inherent limitation of old LAMP primer set, it missed four microscopically positive samples. Any significant mutation in the extremes of the primer binding site

might contribute to this phenomenon. On the other hand, the fact that one negative sample was a false-positive for *Plasmodium falciparum* using this primer set may be due to rare cross-reactivity of the primers. The factors discussed here might also contribute to such variations in outcome of the same primer set in the different studies mentioned above.

The old LAMP method developed by using Poon's [rimer set (Poon *et al.* 2006) had failed to detect five real-time PCR positive samples despite the pre-addition of HNB for easy visual detection while four of them were positive by microscopy also. Three of these four samples had parasitemia of 16 parasites/µL. However, one sample found positive only by real-time PCR crossed the detection threshold in last cycle of the real-time PCR. The present also confirmed that the primer set described by Poon *et al.* (2006) can detect a minimum of 100 parasites/µL.

Sensitivity and specificity

Poon *et al.* (2006) described 95% sensitivity and 99% specificity in regard to PCR in their first implementation of LAMP in malaria. The same primer set was replicated in the genus-species specific LAMP described by Han *et al.* (2007), where all of the 12 microscopy and PCR positive *P. falciparum* samples were successfully detected by LAMP. However, Poschl *et al.* (2010) described 100% sensitivity and specificity of LAMP compared to nested PCR using the same primer set and reaction conditions. Lucchi *et al*. (2010) have also utilized the same primer set in real-time fluorescence LAMP with portable fluorescence reader. That study found 98.8% sensitivity with 100% specificity of the method while Paris *et al.* (2007) found 73.1% sensitivity compared to microscopy using same primer set with specificity 100% in their study.

Yamamura *et al.* (2009) designed another 18S rRNA based primer set for *P. falciparum* detection, which can detect 10 copies of plasmid DNA after 80 minutes of amplification. This method was determined to be as 97.8% sensitive and specificity was found to be 85.7%. However, cross reactions of the primers with *P. vivax* and other undefined targets was ignored by melt curve analysis. Their method also required sophisticated instruments like Genopattern Analyzer GP1000 which limits wide application of the method (Yamamura *et al*. 2009).

Polley *et al.* (2010) described another primer set targeting mitochondrial DNA for detection of *P. falciparum* malaria. Their primer set also could detect 5 parasites/µL. Their primer set was 93.3% sensitive and 100% specific to identify *P. falciparum* cases. In recent time, a commercial Pan/Pf LAMP assay was developed and evaluated in two different settings (Hopkins *et al.* 2013; Polley *et al.* 2013). Both of these studies reported highly sensitive detection of malaria cases but one study (Hopkins *et al.* 2013) reported compromised specificity and NPV when implemented in the field level. This might be due to the detection of a high number of false positive samples. The kit used expensive calcein or turbidity meter. Calcein fluorescence has some drawbacks also such as lower detection sensitivity and brightness of the fluorescence as observed in a previous study (Goto *et al*. 2009).

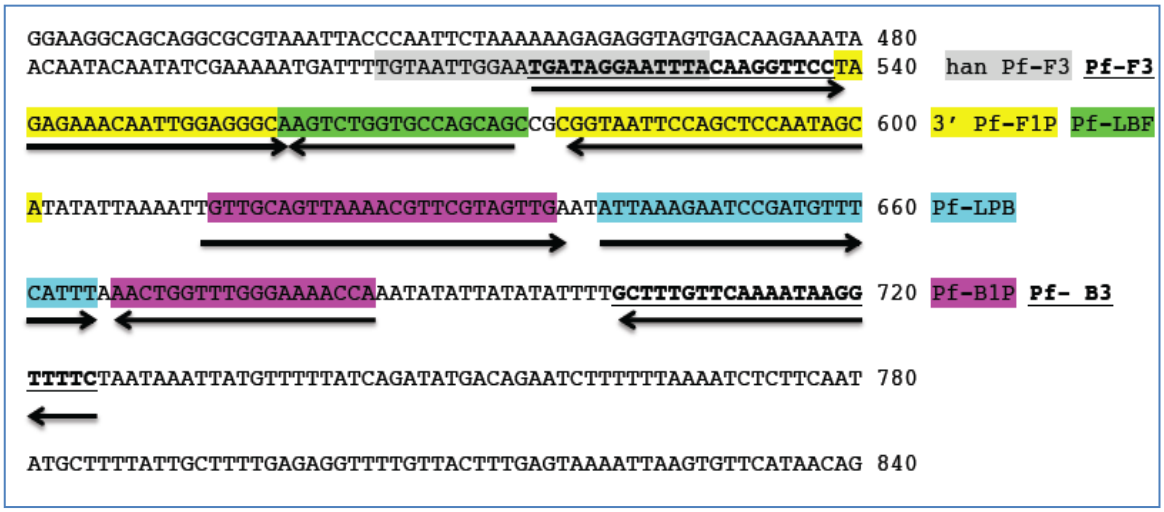


Figure 6.6: The newly designed forward primer begins after the initial 11 nucleotides of the Poon's forward primer in gray. The reverse primers Pf-B3 are identical. The other new primers which are underlined are shifted from the Poon's corresponding primers by 4-10 nucleotides.

Introducing easier sample preparation rather than Qiagen extraction with the sensitive and specific primer set and simple and cheap endpoint visual detection developed by the study might provide a practical way to develop a LAMP assay for *P. falciparum* for routine diagnostic purposes in the field level. The newly developed LAMP method had very good concordance with microscopy and real-time PCR was 0.98 in both cases in a scale of 1.0. However, this value for old LAMP by Poon *et al.* (2006) was 0.94. High positive predictive value of the test method indicates its ability to detect actual positive cases. Conversely, high negative predictive values reveal its accuracy in excluding false-positive cases which is also reflected in the 100% specificity of the new LAMP method.

Finally, this can be stated that the newly developed LAMP method is highly sensitive and specific for diagnosis of symptomatic *falciparum* malaria. This method can be an alternative molecular diagnostic tool to PCR and might become a standard method for wide use. Furthermore, this method has immense potential to be turned into a tangible tool for point of care diagnosis of malaria and treatment monitoring in healthcare and epidemiological studies.

CHAPTER 7

GENETIC DIVERSITY OF *PLASMODIUM FALCIPARUM*

Material and Methods

Sampling Area

The study samples were collected from 7 malaria endemic districts (Bandarban, Rangamati, Khagrachari; Netrokona, Mymensingh, Sylhet and Cox's Bazar) of Bangladesh. Out of these 7 districts 3 were from Chittagong Hill Tracts region (Bandarban, Rangamat and Khagrachari), here after referred as **CHT** areas and 4 were from other endemic areas (Netrokona, Mymensingh, Sylhet and Cox's Bazar) here after referred as **non-CHT** areas (Figure 7.1).

Sample collection

Febrile patients referred to microscopy for malaria diagnosis of several Upazila Health Complexes of those seven selected districts from July 2010-May 2013 followed by confirmation of *Plasmodium falciparum* mono infection by microscopy, nested PCR and/or real-time PCR were enrolled. After confirmation of *P. falciparum* mono infection by microscopy the samples were then preserved in EDTA tube and stored at -20°C in the field site. DNA extraction and molecular diagnosis were carried out at the Parasitology Laboratory of icddr,b.

DNA extraction

DNA was extracted from 200µL preserved whole blood using QiaAmp blood mini kit (Qiagen GmbH, Germany) following manufacturer's instructions. Detail process of DNA extraction has been described in a previous section (chapter 5).

Nested PCR

Nested PCR was done on extracted DNA according to the procedure described by Snounou *et al.* (1993b). The nested PCR targets the 18sr-RNA gene of *P. falciparum* amplifying 1.2kb fragment using a pair of primer. Then in the second step, 1 µl diluted amplicon from the 1st step was used as template. The expected band size for *P. falciparum* was 205 bp.

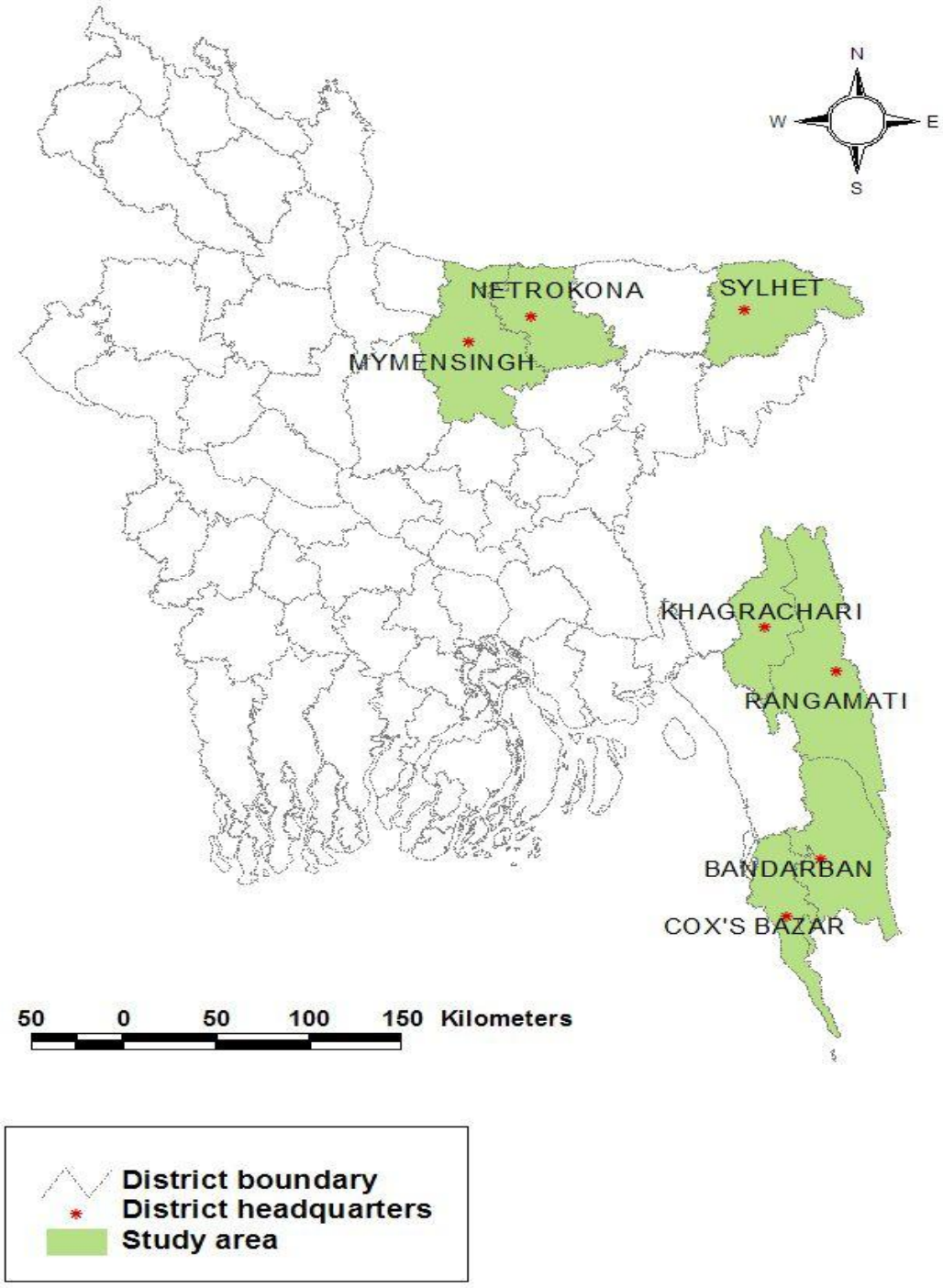


Figure 7.1: Study areas for *Plasmodium falciparum* genotyping study.

Real-time PCR

Real-time PCR was done on extracted DNA according to the procedure described in a previous section (chapter 5) using Invitrogen™ SYBR Green I supermix UDG (Life Technologies Corporation, USA) in a Thermocycler (MyCycler™, Biorad Laboratories, Inc) (Figure: 7.2).

Genotyping of *Plasmodium falciparum*

PCR amplification of template DNA and analysis of region II of GLURP, central polymorphic region of MSP2 (3D7/IC and Fc27 allelic families), and block 2 of MSP1 (K1, MAD20 and RO33 allelic families) was performed followed by the method described by the Snounou *et al.* (1999).

Primers

A total of 18 primers were used for genotyping of *P. falciparum*. Detail list of primers are provided in the Tables 7.1 to7.3 based on allelic families separately.

Positive controls

The positive controls of targeted allelic family of MSP-1 (K1, MAD20 and RO33 of block 2), MSP-2 and GLURP for the study were obtained from the Swiss Tropical and Public Health Institute (STPHI). The positive control samples were provided in filter papers from the *P. falciparum* trophozoite cultures. DNA was extracted from these filter papers at the Parasitology laboratory of icddr,b by using the method described in an earlier section (chapter 5).

Polymerase Chain Reaction (PCR) for MSP1

Purified DNA template was used for amplification using specific primer sets for MSP1 as described by Snounou *et al.* (1999). In the nested-PCR based method, purified DNA templates were amplified using a MyCycler thermal cycler (Biorad) with a species-specific primer set in the first step of the nested PCR (Nest 1). The diluted products from Nest 1 (Primary product) was used for the second step allele specific PCR (Nest 2). Positive and negative controls were systematically incorporated in each PCR run.

Table 7.1: Sequences of the primers used to genotype MSP1 allelic families of *P. falciparum*.

Primer	Sequence	Notes
M1-OF	5'-CTAGAAGCTTTAGAAGATGCAGTATTG-3'	Conserved- Nest 1
M1-OR	5'-CTTAAATAGTATTCTAATTCAAGTGGATCA-3'	Conserved- Nest 1
M1-KF	5'-AAATGAAGAAGAAATTACTACAAAAGGTGC-3'	K1 family specific-Nest 2
M1-KR	5'-GCTTGCATCAGCTGGAGGGCTTGCACCAGA-3'	K1 family specific-Nest 2
M1-MF	5'-AAATGAAGGAACAAGTGGAACAGCTGTTAC-3'	MAD20 family specific-Nest 2
M1-MR	5'-ATCTGAAGGATTTGTACGTCTTGAATTACC-3'	MAD20 family specific-Nest 2
M1-RF	5'-TAAAGGATGGAGCAAATACTCAAGTTGTTG-3'	RO33 family specific-Nest 2
M1-RR	5'-CATCTGAAGGATTTGCAGCACCTGGAGATC-3'	RO33 family specific-Nest 2

2µl of purified DNA template was used for the first step of the nested PCR (Nest 1) with 300nM of each primer, 200µM each of four dNTPs, 3.5 mM $MgCl_2$, 1X BSA and 1.25U of Taq DNA Polymerase (New England Biolabs, Inc.) in a total volume of 25µl reaction mixture. The cycle conditions for the Nest 1 step with an initial denaturation period of 3 min at 94º C were: 35 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min, extension at 72°C for 1 min 30 sec and final extension for 10 min to ensure that all product were in full-length.

The polymorphic repetitive regions block 2 of MSP-1 was amplified by nested PCR. The second amplification reactions (Nest 2) were based on the primary products (products from Nest 1) using allelic-specific primers sets corresponding to K1, RO33 and MAD20 families of MSP-1. The primary products were diluted 6 times in deionized water.

2µl of primary product (from Nest 1) was used for Nest 2 as template with 300 nM of each family specific primer, 200µM each of four dNTPs (Invitrogen), 3.5 mM $MgCl_2$, 1X BSA and 0.75U of Taq DNA Polymerase (New England Biolabs, Inc.) in a total volume of 25µl reaction mixture for the 3 different family specific Nest 2. The cycle conditions for the Nest 2 step with an initial denaturation period of 3 min at 94º C were: 35cycles of denaturation at 94°C for 1 min, annealing at 61°C for 1 min, extension at 72°C for 1 min and final extension for 5 min to assure that all product were in full-length.

Reaction Mixture (For 25µl):

Nest 1:

10X NEB Standard Taq Buffer	2.5 µl
2mM NEB dNTPS	2.5 µl (final concentration 200 µM each)
25mM NEB $MgCl_2$	2.0 µl (final concentration 3.5 µM)
10 µM M1-F (primer)	0.75 µl (final concentration 300 nM)
10 µM M1-R (primer)	0.75 µl (final concentration 300 nM)
NEB Taq polymerase	0.25 µl (1.25 U)
100X BSA	0.25 µl
dH_2O	14.00 µl
Template DNA	2.0 µl

Nest 2 (for K1, MAD20, RO33):

10X NEB Standard Taq Buffer	2.5 µl
2mM NEB dNTPS	2.5 µl (final concentration 200 µM each)
25mM NEB $MgCl_2$	2.0 µl (final concentration 3.5µM)
10 µM M1-KF/M1-MF/M1-RF (primer)	0.15 µl (final concentration 300 nM)
10 µM M1-KR/M1-MR/M1-RR (primer)	0.15 µl (final concentration 300 nM)
NEB Taq polymerase	0.15 µl (0.75 U)
100X BSA	0.25 µl
dH_2O	15.30 µl
Diluted Primary product	2.0 µl

Figure 7.2: Thermal Cycler used in the present study.

Polymerase Chain Reaction (PCR) for MSP2

The nested PCR based method described by Snounou *et al.* (1999) was followed for amplification of central polymorphic region of MSP-2. Purified DNA templates were amplified in a MyCycler thermal cycler (Figure 7.2) with a species-specific primer set in the first step of the nested PCR (Nest 1). The 6 times diluted products from Nest 1 (Primary product) was used for the second step allele specific PCR (Nest 2). Positive and negative controls were methodically incorporated in each PCR run.

2µl of purified DNA template was used for the first step of the nested PCR (Nest 1) with 120nM of each primer, 200µM each of the four dNTPs (Invitrogen), 3.5mM $MgCl_2$, 1X BSA and 1.25U of Taq DNA Polymerase (New England Biolabs Inc.) in a total volume of 25µl reaction mixture. The cyclic conditions for the Nest 1step with an initial denaturation period of 3 min at 94º C were: 35 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min, extension at 72°C for 1 min 30 sec and final extension for 5 min to ensure that all product were in full-length.

The polymorphic repetitive regions block 2 of MSP-2 was amplified by nested PCR. The second amplification reactions (Nest 2) were based on the primary products (products from Nest 1) using allelic-specific primers sets corresponding to Fc27 and 3D7/IC families of MSP-2. The primary products were diluted 6 times in deionised water.

2µl of diluted primary product (from Nest 1) was used for Nest 2 as template with 120nM of each primer, 200µM each of the four dNTPs (Invitrogen), 3.5mM $MgCl_2$, 1X BSA and 0.8U of Taq DNA Polymerase (New England Biolabs Inc.) in a total volume of 25µl reaction mixture for the 2 different family specific Nest 2. The cycle conditions for the Nest 2 step with an initial denaturation period of 3 min at 94º C were: 35 cycles of denaturation at 94°C for 1 min, annealing at 61°C for 1 min, extension at 72°C for 1 min and final extension for 5 min to assure that all product were in full-length.

Table 7.2: Sequences of the primers used to genotype MSP2 allelic families of *P. falciparum*.

Primer	Sequence	Notes
M2-OF	5′- ATG AAG GTA ATT AAA ACA TTG TCT ATT ATA-3′	MSP2 Conserved- Nest 1
M2-OR	5′- CTT TGT TAC CAT CGG TAC ATT CTT-3′	MSP2 Conserved- Nest 1
M2-Fc27F1	5′-AA T ACT AAG AGT GTA GGT GCA AAT GCT CCA-3′	Fc27 family specific-Nest 2
M2-Fc27F2	5′-AAT ACT AAG AGT GTA GGT GCA GAT GCT CCA-3	Fc27 family specific-Nest 2
M2- Fc27R	5′- TTT TAT TTG GTG CAT TGC CAG AAC TTG AAC-3′	Fc27 family specific-Nest 2
M2-3D7/ICF	5′- AGA AGT ATG GCA GAA AGT AAG CCT CCT ACT-3′	3D7/IC family specific-Nest 2
M2-3D7/ICR	5′- GAT TGT AAT TCG GGG GAT TCA GTT TGT TCG-3′	3D7/IC family specific-Nest 2

Reaction Mixture (For 25µl):

Nest 1:

10X NEB Standard Taq Buffer	2.5 µl
2mM NEB dNTPS	2.5 µl (final concentration 200µM each)
25mM NEB $MgCl_2$	2.0 µl (final concentration 3.5µM)
10 µM M2-OF (primer)	0.3 µl (final concentration 120 nM)
10 µM M2-OR (primer)	0.3 µl (final concentration 120 nM)
NEB Taq polymerase	0.25 µl (1.25 U)
dH_2O	15.95 µl
Template DNA	2.0 µl

150 µl dH_2O added to the primary product.

Nest 2 (for FC27):

10X NEB Standard Taq Buffer	2.5 µl
2mM NEB dNTPS	2.0 µl (final concentration 200µM each)
25mM NEB $MgCl_2$	2.0 µl (final concentration 3.5µM)
10 µM M2-Fc27F1 (primer)	0.3 µl (final concentration 120 nM)
10 µM M2-Fc27F2 (primer)	0.3 µl (final concentration 120 nM)
10 µM M2Fc27R (primer)	0.3 µl (final concentration 120 nM)
NEB Taq polymerase	0.2 µl (0.8 U)
100X BSA	0.25 µl

dH_2O	15.15 µl
Diluted Primary product	2.0 µl
Nest 2 (for 3D7/IC):	
10X NEB Standard Taq Buffer	2.5 µl
2mM NEB dNTPS	2.5 µl (final concentration 200µM each)
25mM NEB $MgCl_2$	2.0 µl (final concentration 3.5µM)
10 µM M2-3D7/ICF (primer)	0.3 µl (final concentration 120 nM)
10 µM M2-3D7/ICR (primer)	0.3 µl (final concentration 120 nM)
NEB Taq polymerase	0.2 µl (0.8 U)
100X BSA	0.25 µl
dH_2O	15.15 µl
Diluted Primary product	2.0 µl

Polymerase Chain Reaction (PCR) for GLURP

A semi-nested PCR approach described by Snounou *et al.* (1999) was adopted to amplify the region II of GLURP. Purified DNA templates were amplified in a MyCycler thermal cycler (Biorad) with a species-specific primer set in the first step of the nested PCR (Nest 1). The diluted products from the Nest 1 (primary product) were used for the second step PCR (NEST 2). Positive and negative controls were systematically incorporated in each PCR run.

In the first step of the semi-nested PCR, 2 µL of purified DNA was amplified with 100nM of each primers, 150 µM each of four dNTPs (Invitrogen), 3.5 µM $MgCl_2$, 1X BSA and 0.8U of Taq DNA polymerase (New England Biolabs, Inc.). The product from the first step of the PCR was diluted 5 times with deionized water and 1 µL was used for the second step. The second step of the PCR was done with 100nM of each primer, 200 µM each of four dNTPs (Invitrogen), 3.5 µM $MgCl_2$, 1X BSA and 0.8U of Taq DNA polymerase (New England Biolabs, Inc.). The cycle conditions for the both step with an initial denaturation period of 3 min at 95º C were: 35 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min, extension at 68°C for 2 min and final extension for 5 min.

Table 7.3: Sequences of the primers used to genotype GLURP allelic families of *P. falciparum*.

Primer	Sequence	Notes
G-OF	5′- TGA ATT TGA AGA TGT TCA CAC TGA AC-3′	GLURP Conserved- Nest
G-OR	5′- GTG GAA TTG CTT TTT CTT CAA CAC TAA-3′	GLURP Conserved- Nest 1 and 2
G-NF	5′- TGT TCA CAC TGA ACA ATT AGA TTT AGA TCA-3′	GLURP Conserved- Nest 2

Reaction Mixture (For 20µl):

Nest 1:

10X NEB Standard Taq Buffer	2.0 µl
10mM NEB dNTPS	0.3 µl (final concentration 150 µM each)
25mM NEB $MgCl_2$	2.0 µl (final concentration 3.5µM)
100X BSA	0.2 µl
10 µM G-OF (primer)	0.2 µl (final concentration 100 nM)
10 µM G-OR (primer)	0.2 µl (final concentration 100 nM)
NEB Taq polymerase	0.2 µl (0.8 U)
dH_2O	12.9 µl
Template DNA	2.0 µl

80 µl dH_2O added to the primary product.

Nest 2:

10X NEB Standard Taq Buffer	2.0 µl
10mM NEB dNTPS	0.4 µl (final concentration 200 µM each)
25mM NEB $MgCl_2$	2.0 µl (final concentration 3.5µM)
100X BSA	0.2 µl
10 µM G-NF (primer)	0.2 µl (final concentration 100 nM)
10 µM G-OR (primer)	0.2 µl (final concentration 100 nM)
NEB Taq polymerase	0.2 µl (0.8 U)
dH_2O	13.8 µl
Diluted Primary product	1.0 µl

Gel electrophoresis

The amplified second PCR products for all the markers were either stored at +4°C or analysed immediately by electrophoresis on a 1.5% agarose gel respectively, stained with ethidium-bromide and visualized under UV transillumination (GelDoc®, Biorad,

USA). The sizes of the PCR products were estimated using 25 base pairs (bp) (for MSP-1), 100 and 50 bp (for MSP-2) and 100bp and 1Kb (for GLURP) DNA ladder marker (Sigma-Aldrich Inc., USA).

Statistical Analysis

Data were analyzed by using the SPSS® v16.0 Software (SPSS Inc.). The prevalence of each allelic family was estimated by calculating the percentage of fragments assigned to one family (by PCR with family-specific primers) within the overall number of fragments detected for that locus in the group considered. Comparisons of the distribution of MSP-1, MSP-2 and GLURP allelic families according to areas were made using chi-square tests.

The Multiplicity of infection (number of bands per infected person), a quantitative variable, was calculated as the average number of distinct fragments per PCR positive sample. It was estimated by dividing the total number of fragments detected in the typing reaction by the number of positive samples for that reaction.

Multiplicity of Infection (MOI) = $\frac{Number\ of\ bands\ for\ allele\ 'a'}{Number\ of\ total\ positive\ patients}$

As a measure for genetic diversity, the expected heterozygosity (HE) was calculated by using the formula:

$$HE = [n/(n-1)]\ [(1-\sum Pi2)]$$

Where, n = sample size, P_i = allele frequency. The theoretical probability of infection by two parasites with same allele was calculated as $P = \Sigma P_i^2$, where P_i is the frequency of allele i. This combined probability indicates that two independent clone share the same genotype for all marker genes was calculated by multiplying the probabilities P for all marker genes, assuming that the three loci sort individualistically from each other.

Results and Observations

MSP1 PCR

Out of 130 samples, 111 were positive for MSP1 PCR (85.4%). In CHT areas, 59 out of 69 samples (85.5%) were amplified and in non-CHT areas, 52 out of 61 samples (85.2%) were amplified respectively in MSP1 specific PCR (Table 7.4).

MSP1 Clones

A total of 168 MSP clones were found by MSP1 specific PCR of which 93 (55.4%) were of CHT areas and remaining 75 were of (44.6%) from non-CHT areas. Thus, number of MSP1 clone per positive samples in CHT was 1.57 in CHT areas and 1.44 in non-CHT areas.

MSP1 genotypes per allelic-family/locus

A total 14 alleles were found in Bangladesh by MSP1 specific PCR in three polymorphic regions (K1, MAD20 and RO33). An equal number of 13 allelic families were found in both CHT and non-CHT areas (Table 7.5). However, most of the alleles were common for both the areas.

K1 allele family of MSP1

Out of 63 clones, 7 allele groups of k1 allelic family were recognized from total study samples during the present study (Table 7.5). Of which 6 were common in both the areas. An additional allele from k1 family was found in CHT area. Molecular size varies from 126-300 base pairs in overall and CHT samples and 150-300 base pairs in non-CHT samples (Table 7.6). A typical gel image of K1 family is given in Figure 7.3.

According to frequency in Non-CHT areas, the highest number (10, 15.9%) of clones was found in 201-225 base pair region. On the other hand in CHT areas, the highest number (8, 15.9%) of clones was found in 226-250 base pair region. In overall sample, the highest number (17, 27%) of clones was found in 201-225 base pair region (Figure 7.4-7.5).

Table 7.4: Percentage of different allelic families of MSP1 marker according to study areas.

Area		MSP1			
	No	**K1**	**MAD20**	**RO33**	**Total (%)**
Non-CHT	61	29	27	16	52 (85.2)
CHT	69	30	33	25	59 (85.5)
Total	130	59	60	41	111 (85.4)

Table 7.5: Characteristics of the MSP1 allelic families in the study areas.

Area	K1			MAD			RO33		
	No of Clones	No. Alleles	Mol size (bp)	No of Clones	No. Alleles	Mol size (bp)	No of Clones	No. Alleles	Mol size (bp)
CHT	33	7	126-300	35	5	126-250	25	1	160
Non-CHT	30	6	126-300	29	6	126-325	16	1	160
Total	63	7	126-300	64	6	126-325	41	1	160

Table 7.6: Number of clones according to K1 allelic group of MSP1.

Area	Base Pairs/Allelic group							Total
	126-50	151-75	176-200	201-25	226-50	251-75	276-300	
Non-CHT	0	7	4	10	1	6	2	30
CHT	1	4	4	7	8	7	2	33
Total	1	11	8	17	9	13	4	63

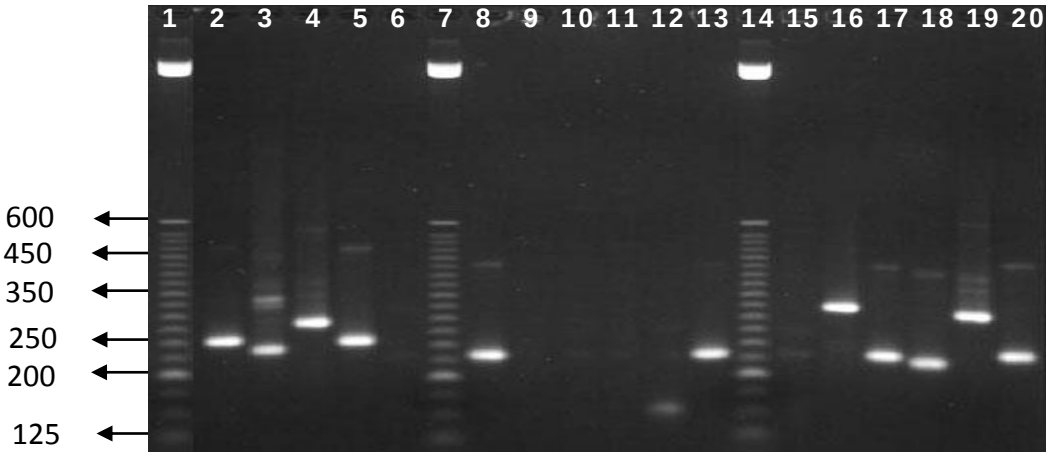


Figure 7.3: Gel image of the PCR products of K1 allelic family. Lane 1, 7, 14: 25 bp ladder, Lane 18: Positive control, Lane 6: Negative control, Lane 2-5, 8-13, 15-17, 19-20: PCR product.

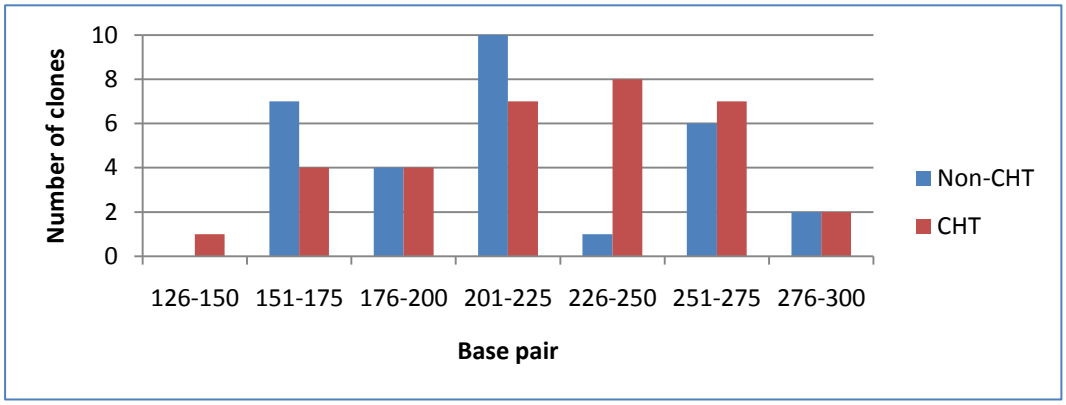


Figure 7.4: Frequency of K1 clones according to study areas.

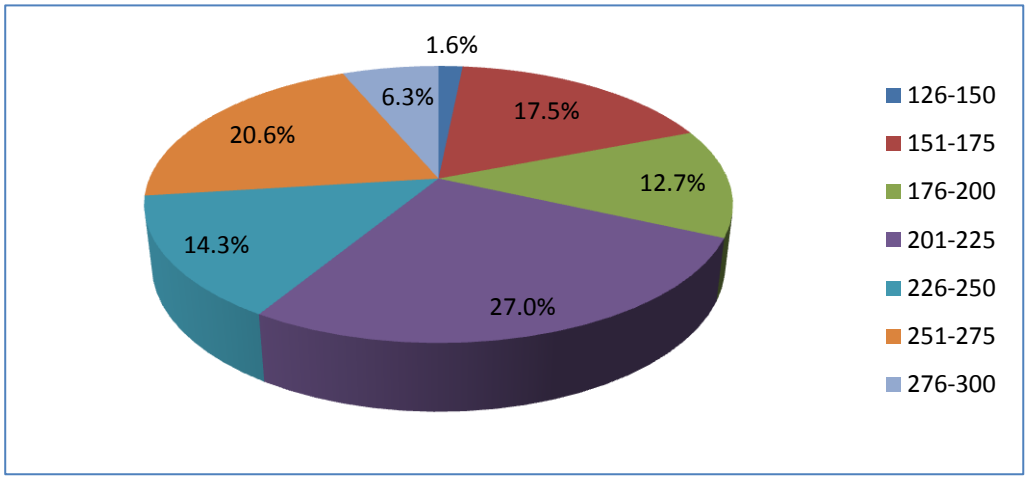


Figure7.5: Percentage of K1 clones frequency according to base pair.

MAD20 allele family of MSP1

Out of 64 clones of MAD20 allelic family, 6 allele groups were recognized from total study samples during the present study (Table 7.5). Of which 5 were common in both the areas. An additional allele from MAD20 family was found in CHT area. Molecular size varies from 126-325 base pairs in overall and Non-CHT samples and 126-250 base pairs in non-CHT samples. A typical gel image of MAD20 family is given in Figure 7.6.

In non-CHT area the highest number of clones (9,14.1%) was found in 176-200 base pair region. Whereas, in CHT areas the highest number of clones (13, 20.3%) was found in two size groups: 151-175 and 176-200 base pair region (Table 7.7). In overall sample the highest number of clones (22, 34.4%) was found in 176-200 base pair region followed by 151-176 base pair group where (21, 32.8%) samples were obtained (Figure 7.6 and 7.7).

RO33 allele family of MSP1

RO33 family is considered as monomorphic. Therefore, out of 41 clones only 1 allele group of RO33 allelic family was recognized from total study samples during the present study (Table 7.5). A typical gel image of RO33 is given in Figure 7.9. The exact molecular size of RO33 was 160 bp and it was found in 16 isolates in non-CHT areas and 25 isolates in CHT areas respectively (Figure 7.10 and 7.11).

Over all frequency of MSP1 allelic group

Being monomorphic clones of RO33 allelic family was found highest (31.5%) in overall distribution. However, the contribution of non-CHT and CHT areas on RO33 frequency distribution were 12.3% and 19.2%, respectively. In K1allelic family, the highest frequency distribution (13.1%) was reported in 201-225 bp categories from overall data. Whereas, the contribution of on k1 frequency distribution reported from non-CHT in 201-225 bp group (7.7%) and from CHT in 226-250 bp group (6.2%), respectively. In MAD20 allelic family, the highest contribution (16.9%) for frequency distribution was reported in 176-200 bp categories from overall data. Whereas, the contribution of on MAD20 frequency distribution reported from non-CHT in176-200 bp group (6.9%) and from CHT in 151-175 and 176-200 bp groups (10% in each), respectively (Figure 7.12).

Table 7.7: Number of clones in MAD20 allelic family of MSP1.

Area	Base Pairs*						Total
	126-50	151-75	176-200	201-25	226-50	301-325	
Non-CHT	3	8	9	6	2	1	29
CHT	1	13	13	6	2	0	35
Total	4	21	22	12	4	1	64

* Base pair groups 251-275 and 276-300 were not found in the present study

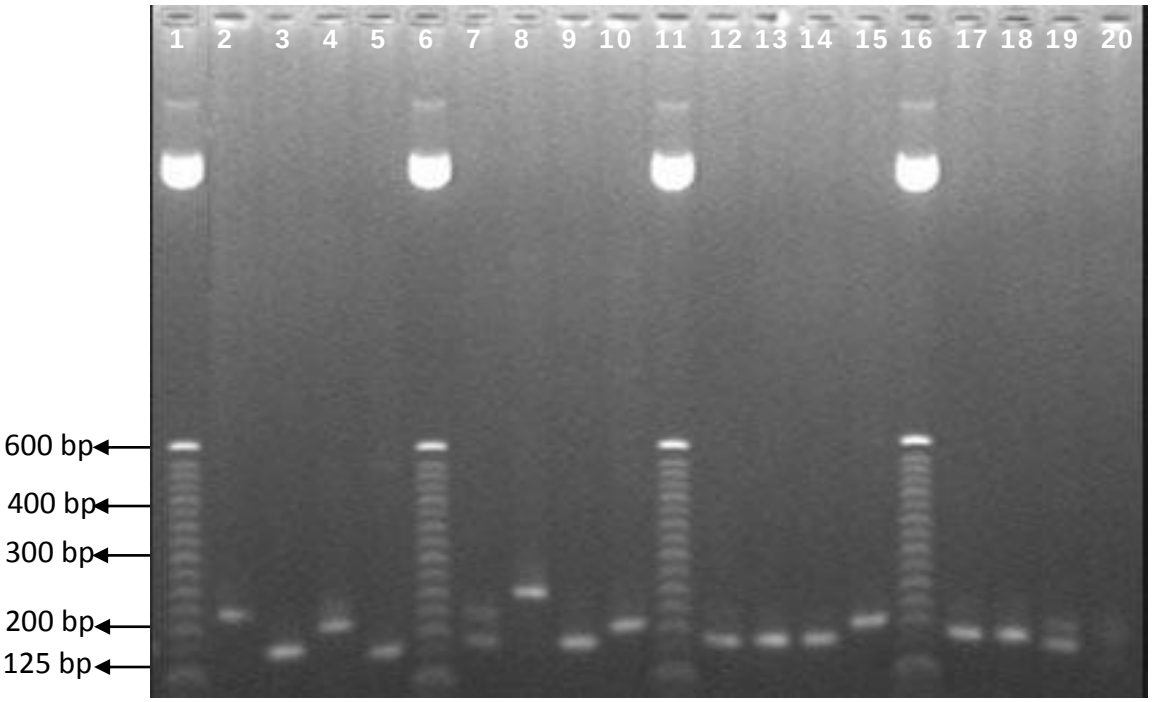


Figure 7.6: Gel image of the PCR product for the MAD20 allelic family. Lane 1, 6, 11, 16: 25 bp ladder, Lane 2: Positive control, Lane 3-5, 7-10, 12-15, 17-19: PCR product, Lane 20: Negative control.

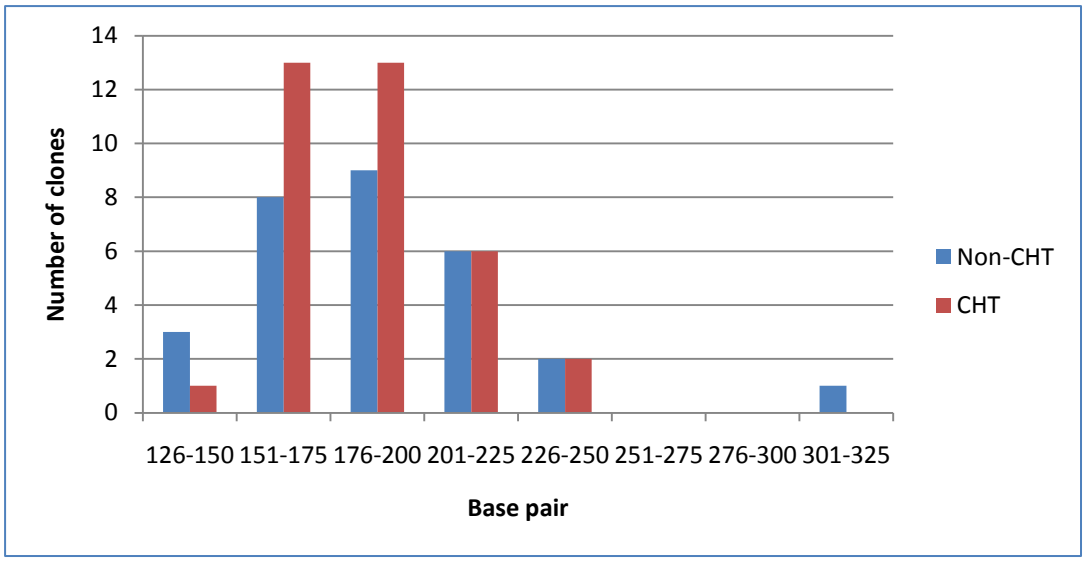


Figure 7.7: Frequency of MAD20 clones according to study areas.

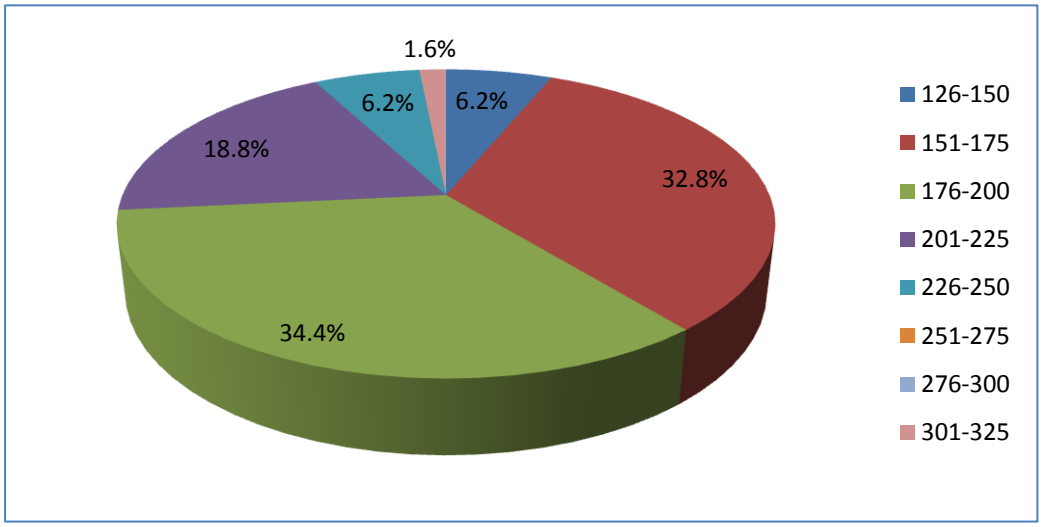


Figure 7.8: Percentage of MAD20 clone frequency according to base pair.

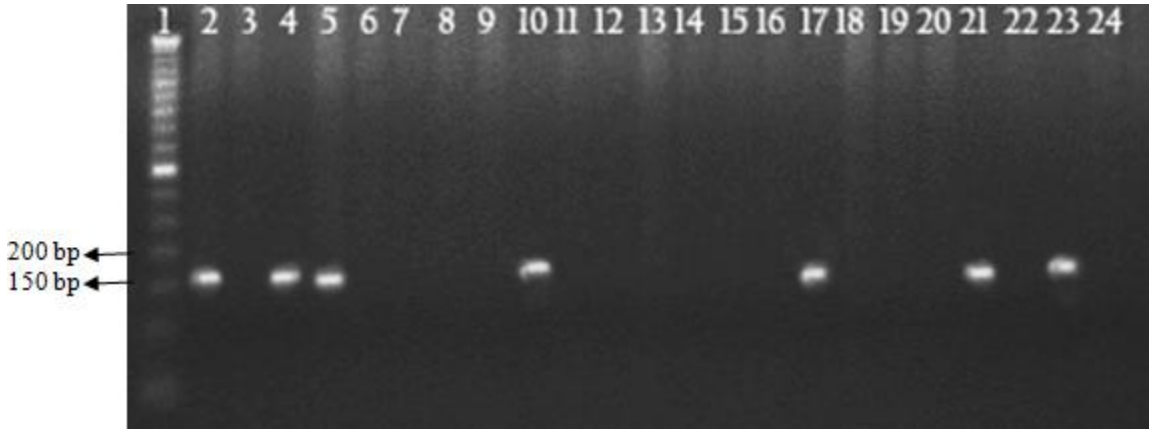


Figure7.9: Gel image of the PCR product for RO33 allelic family. Lane 1:50 bp ladder, Lane 2: Positive control, Lane 4, 5, 10, 21, 23: PCR product, Lane 3: Negative control.

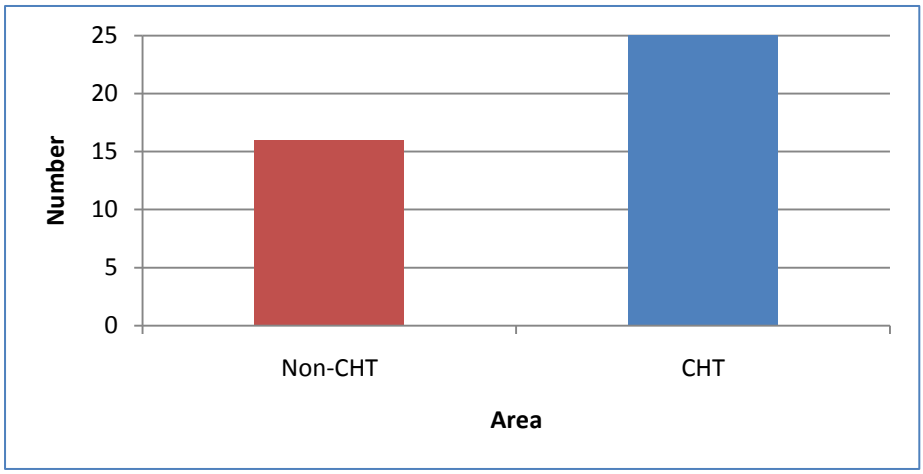


Figure 7.10: Frequency of the RO33 clone according to study areas.

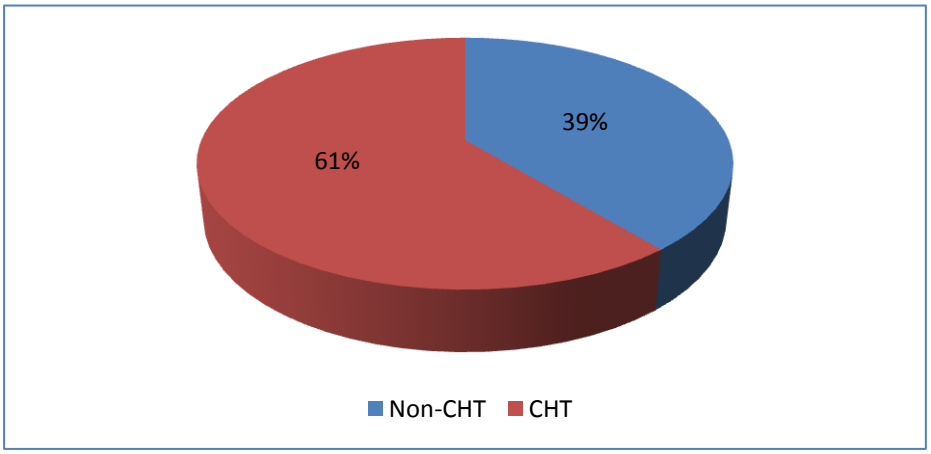


Figure 7.11: Percentage of RO33 clone frequency according to study areas.

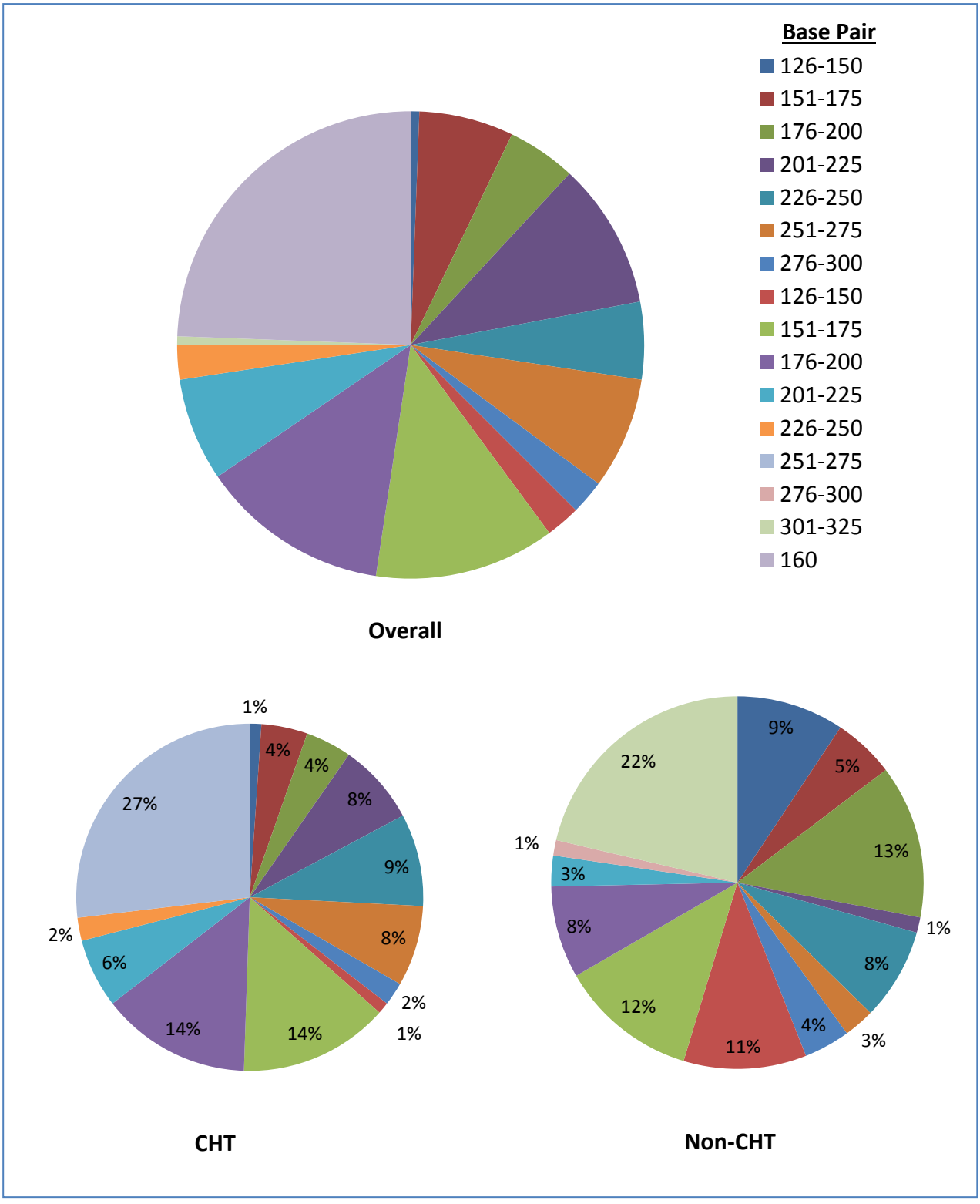


Figure 7.12: MSP1 allelic diversity (%) in overall and according to region.

MSP2 PCR

Out of 130 samples, 102 were positive for MSP2 PCR (78.5%). In CHT areas, 51 out of 69 samples (73.9%) and in non-CHT areas, 51 out of 61 samples (83.6%) contained MSP2 clones (Table 7.8).

MSP2 Clones

A total of 128 clones were found by MSP2 specific PCR of which 66 (51.6%) were from non-CHT areas and remaining 62 (48.4%) were from CHT areas (Table 7.8). Thus, number of MSP2 clone per positive samples was 1.29 in non-CHT areas and 1.22 in CHT areas.

MSP2 genotypes per allelic-family/locus

A total 20 alleles were found in Bangladesh by MSP2 specific PCR in two polymorphic regions (Fc27 and 3D7). Number of allelic families was not equal in both areas. In Non-CHT area 14 alleles were found whereas, in CHT areas 16 alleles were found (Table 7.9). However, most of the alleles were common for both the areas.

Fc27 allele family of MSP2

Out of 54 clones, 8 allele groups of Fc27 allelic family were recognized from total study samples during the present study (Table 7.8). Of which 4 were common in both the areas. Molecular size varies from 251-475 base pairs in overall and non-CHT samples and 251-425 base pairs in CHT samples. A typical gel image of Fc27 allelic family is given in Figure 7.13.

According to frequency, in non-CHT area the highest number (11, 20.4%) was found in 276-300 base pair region. On the other hand in CHT areas the highest number (11, 20.4%) of clones w found in 301-325 base pair region. In overall sample, the highest number (17, 31.5%) of clones was found in 276-300 base pair region (Figure 7.14-7.15).

Table 7.8: Percentage of different allelic families of MSP2allelic families according to study areas.

Area		MSP2		Total (%)
	No	Fc27	3D7/IC	
Non-CHT	61	22	37	51(83.6)
CHT	69	31	30	51(73.9)
Total	130	53	67	102(78.5)

Table 7.9: Characteristics of the MSP2 allelic families in the study areas.

Area	Fc27			3D7/IC		
	No of Clones	No. Alleles	Mol size (bp)	No of Clones	No. Alleles	Mol size (bp)
CHT	31	7	251-425	31	9	351-625
Non-CHT	23	5	251-475	43	9	376-675
Over all	54	8	251-475	74	12	351-675

Table 7.10: Number of clones according to the Fc27 allelic family of MSP2

Area	Base pair								Total
	251-275	276-300	301-325	326-350	351-375	376-400	401-425	451-475	
Non-CHT	5	11	0	3	3	0	0	1	23
CHT	1	6	11	5	4	2	2	0	31
Overall	6	17	11	8	7	2	2	1	54

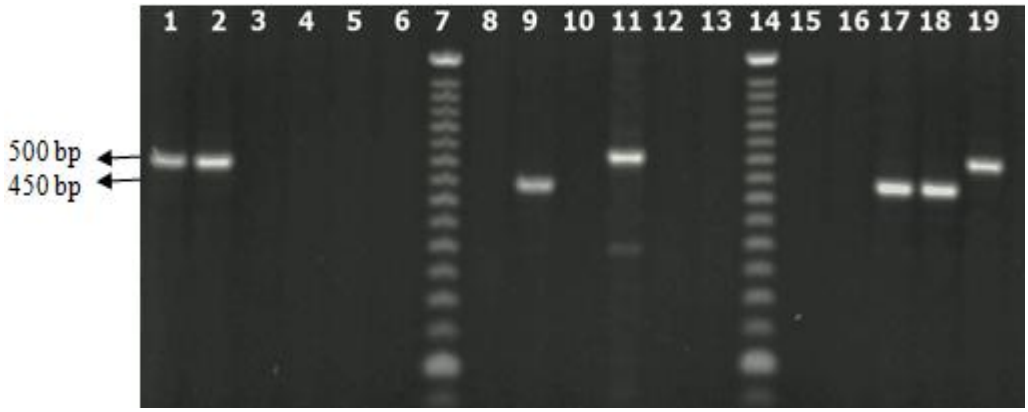


Figure 7.13: Gel image of the PCR product for Fc27 allelic family. Lane 1: Positive control, Lane7 and 14: 50 bp ladder, Lane 2, 9, 17, 18 and 19: PCR product, Lane 15: Negative control.

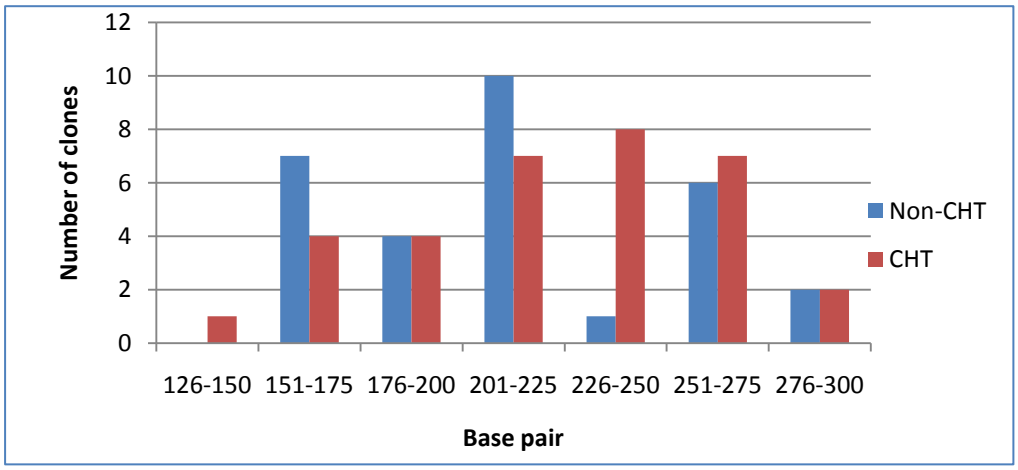


Figure7.14: Frequency of Fc27 clones according to study areas.

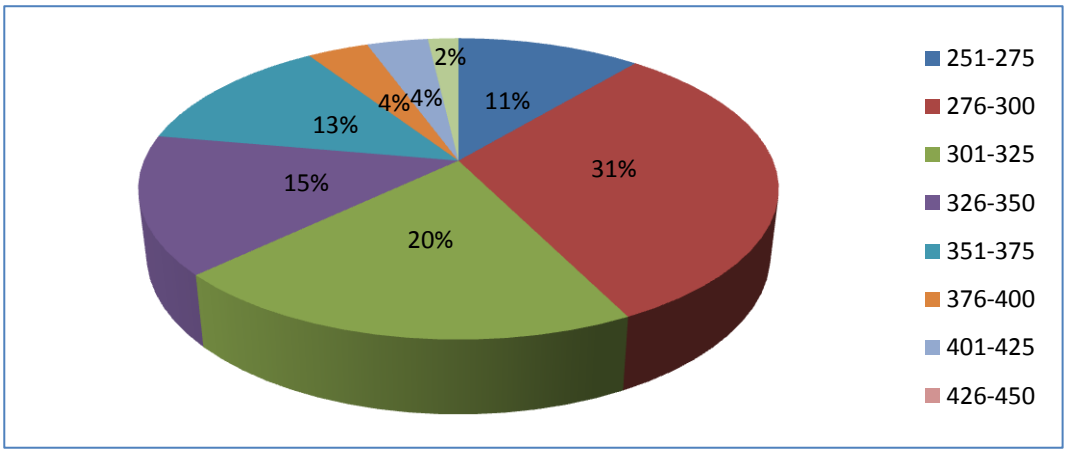


Figure 7.15: Percentage of Fc27clone frequency according to base pair.

3D7/IC allele family of MSP2

Out of 74 clones, 12 allele groups of 3D7/IC allelic family were recognized from total study samples during the present study. Each area had 9 alleles of which 6 were common in both the areas (Table7.9). Molecular size varies from 351-675 base pairs in overall samples, 376-675 in Non-CHT and 351-625 base pairs in CHT samples respectively. A typical gel image of 3D7/IC family is given in Figure 7.16.

According to frequency in Non-CHT area the highest number (11,14.9%) of clones was found in 476-500 base pair region (Table 7.11). Whereas, in CHT areas the highest number of clones (11,14.9%) was found in 501-525 base pair region. In overall sample the highest number of clones (17, 23%) was found in 501-525 base pair region (Figure 7.17-7.18).

Over all frequency of MSP2 allelic group

In Fc27allelic family the highest contribution (13.1%) in frequency distribution was reported in 276-300 bp category from overall data. The highest contribution of Fc27 frequency distribution reported from Non-CHT was also in 276-300 bp group (8.5%) and from CHT in 301-350 bp group (8.5%) respectively. In 3D7/IC allelic family the highest contribution (13.1%) for frequency distribution was reported in 501-525 bp categories from overall data. Whereas, the highest frequency distribution of 3D7/IC was reported from Non-CHT in351-375 bp group (17.7%) and from CHT (8.5%) in 501-525 bp group respectively (Figure 7.19).

Table 7.11: Number of clones in 3D7/IC allelic group of MSP2.

Base Pair	Non-CHT	CHT	Total
351-375	0	1	1
376-400	4	0	4
401-425	0	2	2
426-450	4	0	4
451-475	4	2	6
476-500	11	3	14
501-525	6	11	17
526-550	6	4	10
551-575	4	5	9
576-600	3	2	5
601-625	0	1	1
651-675	1	0	1
Total	43	31	74

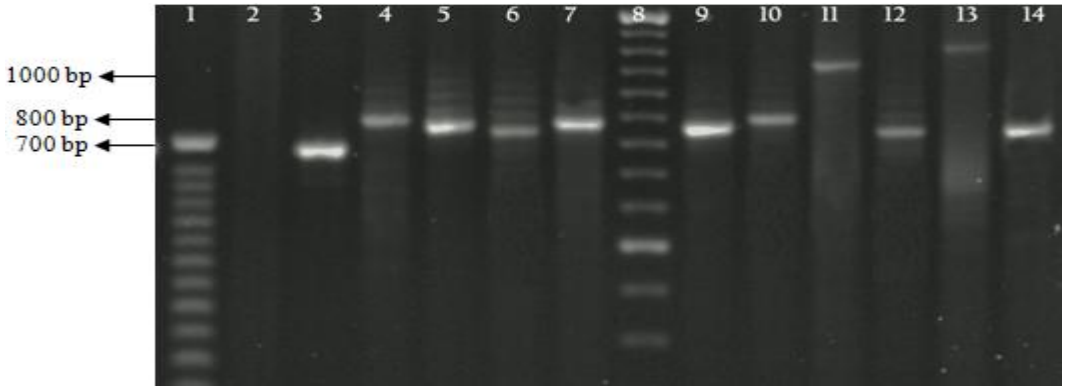


Figure 7.16: Gel image of the PCR product for 3D7/IC allelic family. Lane 1, 8: ladder, Lane 3: Positive control, Lane 4-7 and 9-14: PCR product, Lane 2: Negative control.

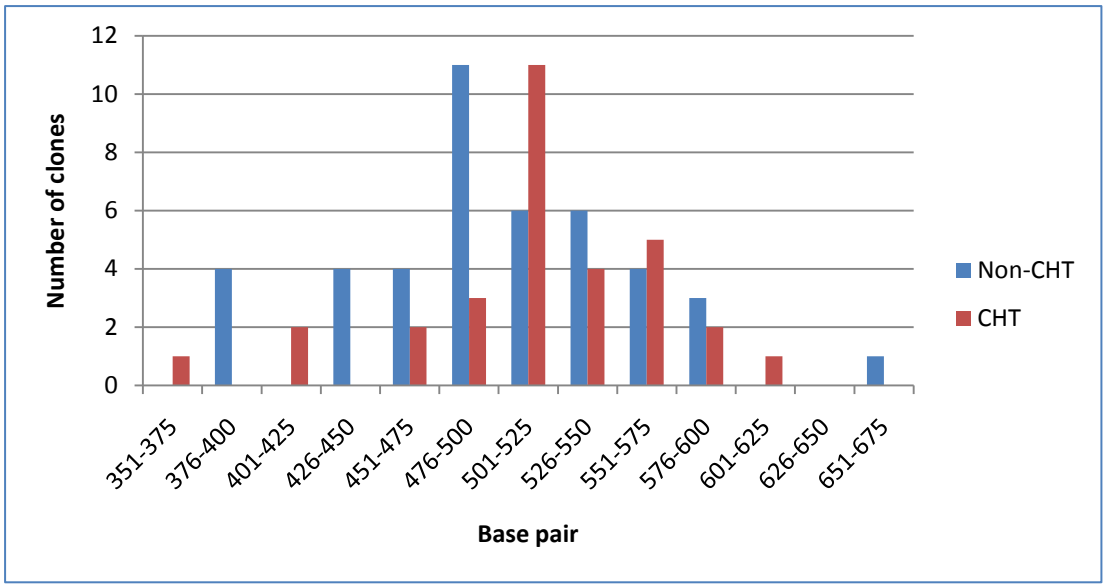


Figure 7.17: Frequency of 3D7/IC clones according to study areas.

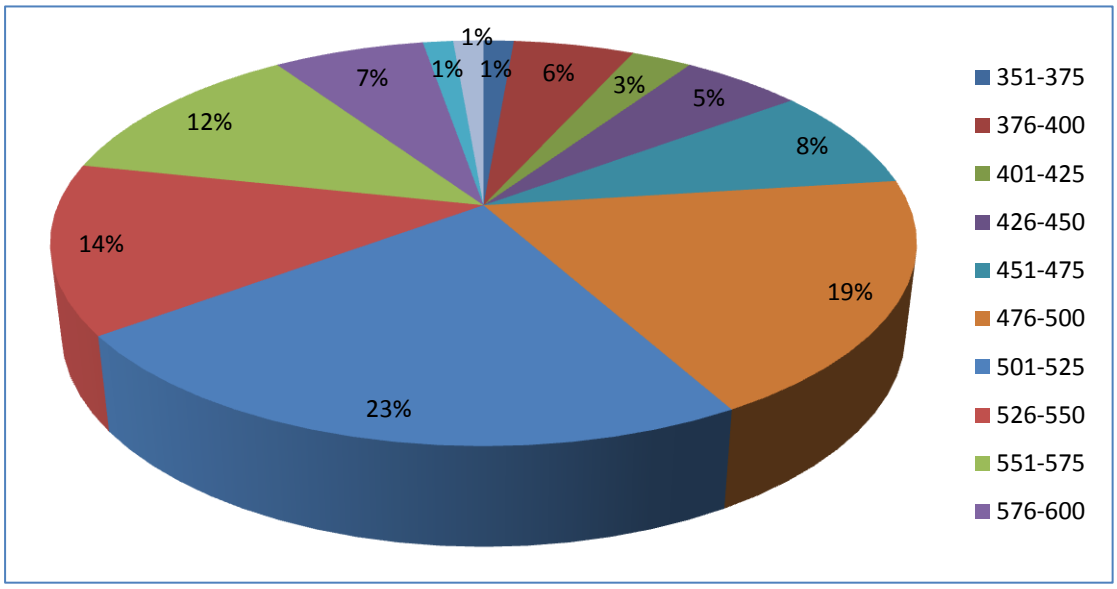


Figure 7.18: Percentage of 3D7/I clone frequency according to base pair.

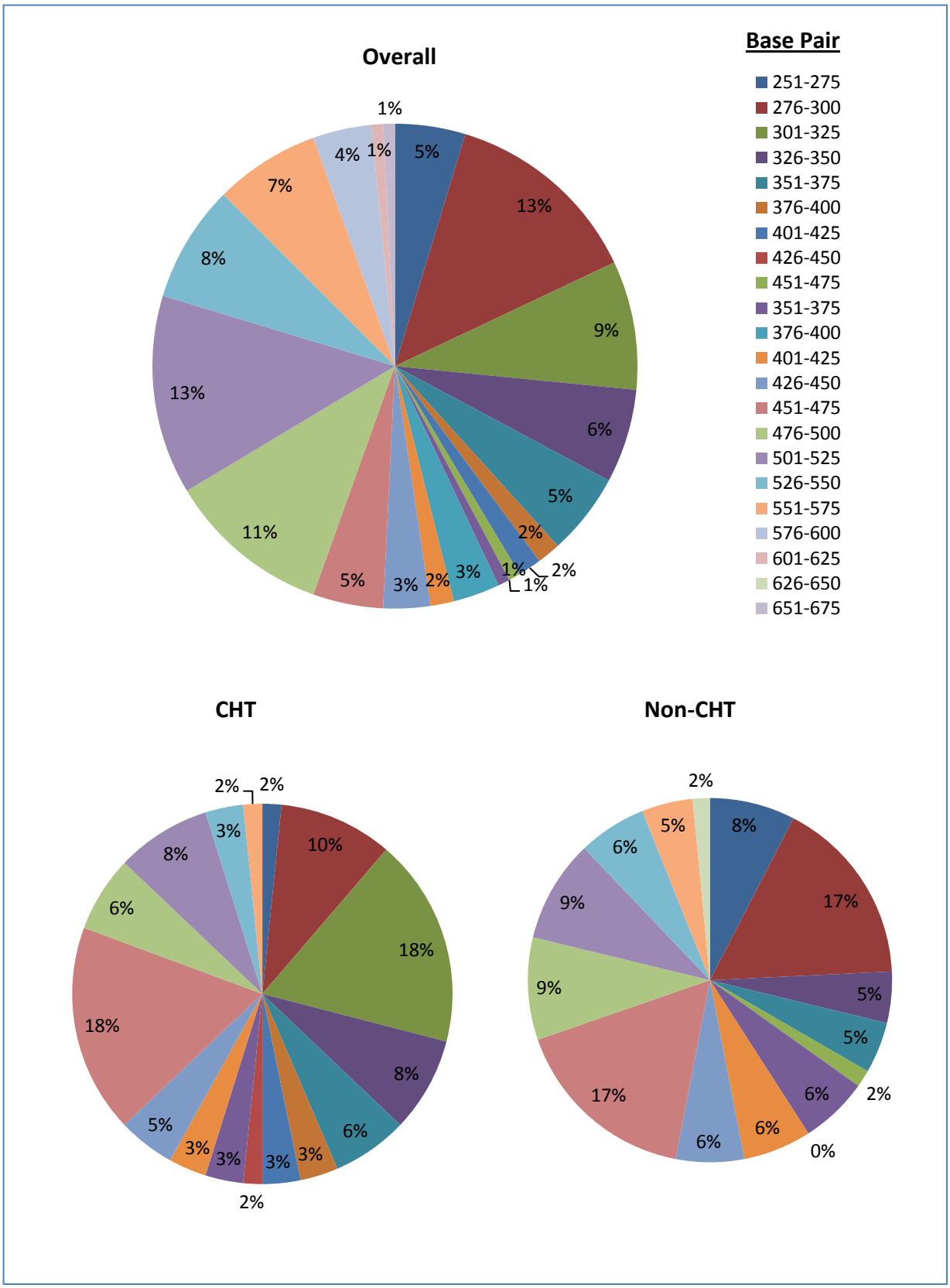


Figure 7.19: MSP2 allelic diversity (%) overall and according to regions.

GLURP PCR

All of the130 samples were positive for GLURP PCR. Thus positivity rate was 100%. A typical gel image of GLURP is given in Figure 7.20.

GLURP Clones

A total of 141clones belonging to 13 allelic families were found by GLURP specific PCR of which 72(51.1%) were from CHT areas and remaining 69 (48.9%) were from non-CHT areas. Thus, number of MSP2 clone per positive samples was 1.04in CHT areas and 1.13 in non-CHT areas. Allelic families were ranged in between 400-1050 bp with highest frequency (26.9%) in 551-600 bp groups in overall data, in non-CHT areas 16.2% and10.8% in CHT along with 901-950 bp group respectively (Table 7.12 and Figure 7.21-7.22).

Table 7.12: Number of clones according to alleles of GLURP

Base Pair	Non-CHT	CHT	Total
400	0	1	1
500	0	1	1
550	1	2	3
600	21	14	35
650	1	2	3
700	6	3	9
750	2	3	5
800	3	8	11
850	4	5	9
900	7	13	20
950	16	14	30
1000	7	6	13
1050	1	0	1
Total	69	72	141

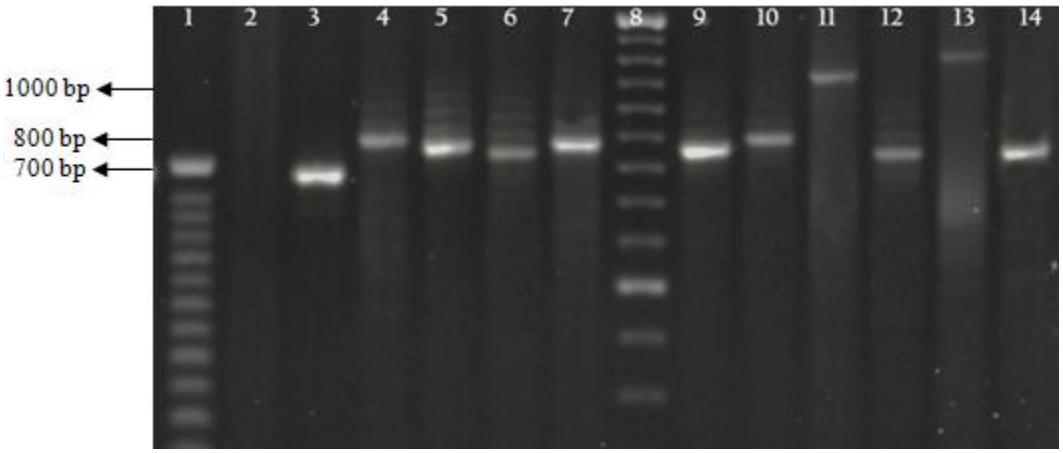


Figure 7.20: Gel image of the PCR product for GLURP. Lane 3: Positive control, Lane1:100 bp ladder, Lane 8: 50 bp ladder, Lane 4-7 and 9-14: PCR product, Lane 2: Negative control.

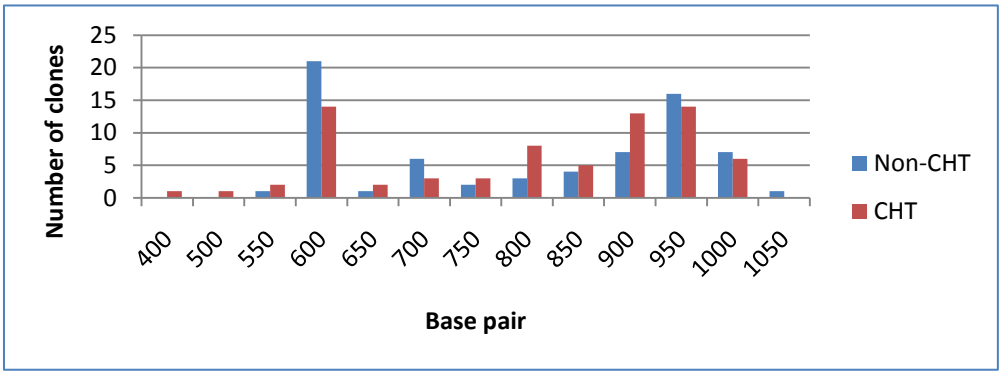


Figure 7.21: Frequency of GLURP clones according to study areas.

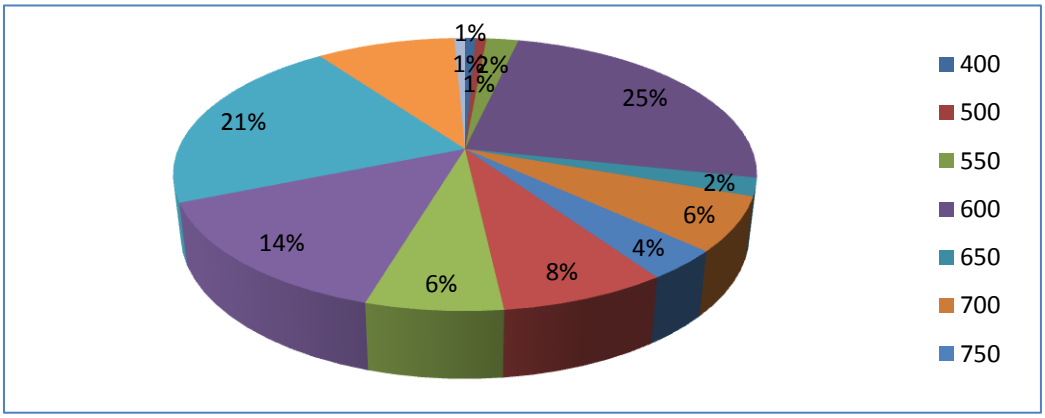


Figure 7.22: Percentage of GLURP clone frequency.

Multiplicity of Infection (MOI)

Mean multiplicity of infection (MOI) and genetic diversity of MSP1, MSP2 and GLURP were calculated following the formula provided in the Material and Methods section. MOI of MSP1, MSP2 and GLURP was 1.51, 1.25 and1.08 respectively in overall samples 1.44, 1.29 and1.11 respectively in non-CHT areas and 1.58, 1.22 and 1.04 respectively in CHT areas (Table 7.13).

The expected heterozygosity (H_E)

The expected heterozygosity (H_E) of MSP1, MSP2 and GLURP were calculated following the formula provided in the Material and Methods section. H_E of MSP1, MSP2 and GLURP in non-CHT areas was found 0.84, 0.90 and0.78 respectively. These values for CHT areas was 0.76, 0.93 and 0.86 respectively and in over all data 0.80, 0.93 and 0.83 respectively (Table 7.13).

Table 7.13: Mean multiplicity of infection (MOI) and expected heterozygosity (H_E) of MSP1, MSP2 and GLURP in study areas.

Area	MSP1		MSP2		GLURP	
	MOI	H_E	MOI	H_E	MOI	H_E
CHT	1.58	0.76	1.22	0.93	1.04	0.86
Non-CHT	1.44	0.84	1.29	0.90	1.11	0.78
BD	1.51	0.80	1.25	0.93	1.08	0.83

Discussion

MSP1 PCR

More than 85% samples were positive for MSP1 PCR during this observation (Table 7.4). The rate is higher than some African countries Tanzania (83%), Malawi (81%), Uganda (76%) and Burkina Faso (53%) observed in a study (Mwingira *et al.* 2011). But less than Senegal (94%) (Konate *et al.* 1999) and Malaysia (87%) (Atroosh *et al.* 2011).

MSP1 Clones

A total of 168 MSP1 clones were found by MSP1 specific PCR in this observation. In Malaysia the 89 MSP1 clones was found out of 65 positive samples (Atroosh *et al.* 2011). Schoepfin *et al.* (2009) found 215 clones in Papua New Guinea and 350 clones in Tanzania out of 108 and 115 samples respectively.

MSP1 genotypes per allelic-family/locus

A total of 14 alleles were found in Bangladesh by MSP1 specific PCR. An equal number of 13 MSP1 allelic families were found in both CHT and non-CHT areas (Table 7.5). In some African countries the number of MSP1 alleles were: 16 in Tanzania, 13 in Malawi , 10 in Uganda, 15 in Burkina Faso (Mwingira *et al.* 2011) and 33 in Senegal (Konate *et al.* 1999). In an observation a total of 14 different alleles of MSP 1 were recognized in Myanmar (Kang *et al.* 2010).

K1 allele family of MSP1

In K1 allelic family 7 alleles were recognized of those 6 were common in both the areas and an additional allele was reported from CHT area (Table 7.5). Mwingira *et al.* (2011) reported 8 alleles from Burkina Faso and Tanzania, 7 from Malawi and 5 from Uganda. Konate *et al.* (1999) reported 10 and 19 alleles from two region of Senegal. Schoepfin *et al.* (2009) reported 13 alleles from Papua New Guinea and 19 from a different population in Tanzania. In recent time Akter *et al.* (2012) reported 6 alleles from K1 family from Bandarban, Bangladesh.

Molecular size varies from 126-300 bp in overall samples for K1 allelic family and CHT samples and 150-300 bp in non-CHT samples. In the present investigation, the highest frequency (27%) was found in 201-225 bp region (Figure 7.4-7.5). This group

was the most frequent in Non-CHT area (15.9%) but 226-250 bp region in CHT areas (15.9%). High frequency in 201-225 and 226-250 bp groups were also observed to be more frequent in some African countries (Mwingira *et al.* 2011) and Schoepfin *et al.* (2009) reported 13 alleles from Papua New Guinea and 19 from a different population in Tanzania.

MAD20 allele family of MSP1

6 allele groups of MAD20 allelic family were recognized from total study samples during the present study, o f which 5 were common in both the areas. An additional allele from MAD20 family was found in CHT area (Table 7.6). Mwingira *et al.* (2011) reported 7 alleles from Tanzania, 6 from Burkina Faso, 5 from Malawi and 4 from Uganda. Konate *et al.* (1999) reported 2 and 12 alleles from two region of Senegal. Schoepfin *et al.* (2009) reported 10 alleles from Papua New Guinea and 9 from a different population in Tanzania. In recent time Akter *et al.* (2012) reported 8 alleles from K1 family from Bandarban, Bangladesh.

Molecular size varied from 126-325 bp with the highest frequency (34.4%) was found in 176-200 bp region in overall samples and also both in CHT (20.3%) and non-CHT areas (14.1%) (Figure 7.6-7.7). High frequency in 176-200 bp group was also dominant in some African countries (Mwingira *et al.* 2011).

RO33 allele family of MSP1

RO33 family is considered monomorphic. Therefore out of 41 clones only 1 allele group of RO33 allelic family was recognized from total study samples during the present study (Table 7.7). The exact molecular size of RO33 was 160 bp as reported by Snounou *et al.* (1999) has been observed in our observation. The monomorphic nature of RO33 was also reported by Mwingira *et al.* 2011in their observation in some Sun-Saharan African countries and by Schoepfin *et al.* (2009) in Papua New Guinea and Tanzania. However, Konate *et al.* (1999) in Senegal, Aubouy *et al.* (2003) in Gabon, Baruah *et al.* (2009) in India and Ghanchi *et al.* (2010) in Pakistan reported polymorphic nature of RO33. Through sequencing Mwingira *et al.* 2011 predicted the amplification size for RO33 in 215 bp region.

Over all frequency of MSP1 allelic group

Due to monomorphic nature, frequency of RO33 allelic family was found highest (31.5%) in overall distribution. In K1allelic family, the highest contribution (13.1%) for frequency distribution was reported in 201-225 bp categories from overall data. Whereas, the contribution of on K1 frequency distribution reported from Non-CHT in 201-225 bp group (7.7%) and from CHT in 226-250 bp group (6.2%) respectively. In MAD20 allelic family the highest contribution (16.9%) for frequency distribution was reported in 176-200 bp categories from overall data. Whereas, the contribution of on MAD20 frequency distribution reported from Non-CHT in176-200 bp group (6.9%) and 10% and from CHT in 151-175 and 176-200 bp groups (10% in each) respectively (Figure 7.12).

MSP2 PCR

Compared to MSP1 less number of isolates was positive for MSP2 in the present observation (102, 78.5%) (Table 7.8). The rate was found lower than some African countries like: Tanzania (93%), Malawi (81%), Uganda (89%) and Burkina Faso (86%) observed in a study (Mwingira *et al.* 2011). This rate was also lower than Senegal (93%) (Konate *et al.* 1999) and Malaysia (33%) (Atroosh *et al.* 2011).

MSP2 Clones

A total of 128 clones were found by MSP2 specific PCR (Table 7.9). In Malaysia out of 25 MSP2 positive samples 20 were found monoclonal and only 5 to be polyclonal (Atroosh *et al.* 2011). Schoepfin *et al.* (2009) found 199 clones in Papua New Guinea and 428 clones in Tanzania out of 108 and 115 samples respectively.

MSP2 genotypes per allelic-family/locus

A total 20 alleles were found in Bangladesh by MSP2 specific PCR in two polymorphic allelic families (Fc27 and 3D7/IC). Mwingira *et al.*(2011) adopted a smaller bin size of 3 bp and thus got high number of MSP2 alleles in some African countries such as: 73 in Tanzania, 69 in Malawi , 49 in Uganda, 97 in Burkina Faso (Mwingira *et al.* 2011). In the same way Schoepflin *et al.* (2009) got 35 alleles in Papua New Guinea and 76 alleles in Tanzania and 33 in Senegal (Konate *et al.* 1999). However, using the same methodology like ours 12 alleles reported by Paul *et al.*

(1998) from Thailand and 19 alleles from Gabon by Aubouy *et al* .(2003) There is no other report of MSP2 genotyping in Bangladesh.

Fc27 allele family of MSP2

In the present study 8 allele groups of Fc27 allelic family were recognized (Table 7.9). Of which 4 were common in both the areas. Mwingira *et al.* (2011) reported 26 alleles from Burkina Faso, 16 from both Malawi and Tanzania, 11 from Uganda Schoepfin *et al.* (2009) reported 8 alleles from Papua New Guinea and 17 from a different population in Tanzania. Konate *et al.* (1999) reported 10 and 11 alleles from two region of Senegal. Ghanchi *et al.* (2010) reported 14 alleles from Pakistan for Fc27 allelic family.

Molecular size for Fc27 allelic family varied from 251-475 bp in the present observation with the highest overall frequency (31.5%) in 276-300 bp regions (Figure 7.14-7.15). Mwingira *et al.* (2011) reported a range of 205-518 bp for occurrence of MSP2 alleles.

3D7/IC allele family of MSP2

A total of 12 allele groups of 3D7/IC allelic family were recognized from total study samples during the present study (Table 7.9). Each area had 9 alleles of which 6 were common in both the areas. Mwingira *et al.* (2011) reported 71 alleles from Burkina Faso, 57 from Tanzania, 53 from Malawi and 38 from Uganda. Schoepfin *et al.* (2009) reported 27 alleles from Papua New Guinea and 59 from a different population in Tanzania. Konate *et al.* (1999) reported 9 and 20 alleles from two region of Senegal. Ghanchi *et al*. (2010) reported 19 alleles from Pakistan for 3D7/IC allelic family and Aubouy *et al.* (2003) reported 8 alleles from Gabon.

Molecular size of clones for 3D7/IC allelic family varied from 351-675 bp in overall samples with the highest frequency (23%) in 501-525 bp region (Figure 7.14-7.15). In Papua New Guinea the frequency of the most common allele was 15.08% and in Tanzania 11.21% respectively (Schoepflin *et al*. 2009).

GLURP PCR

All of the samples (N=130) were positive for GLURP in PCR (100%). Previously Mwingira *et al.* 2011 reported 78% positivity in Uganda, 75% Tanzania and Malawi

and 61% in Burkina Faso. Hadda *et al.* (1999) reported 56% prevalence rate for GLURP in Honduras.

GLURP Clones

A total of 141clones belonging to 13 allelic families were found by GLURP specific PCR. Mwingira *et al.* (2011) reported 14 alleles in Burkina Faso, 13 alleles both in Malawi and Tanzania and 10 alleles from Uganda. Only two alleles of GLURP was reported in Honduras by Hadda *et al.* (1999).

In the present observation the allelic families were ranged in between 400-1050 bp with highest frequency (26.9%) in 551-600 bp groups in overall data, Non-CHT areas 16.2% and10.8% in CHT along with 901-950 bp group respectively (Figure 7.21). Mwingira *et al.* (2011) detected GLURP allelic families in between 650-1250 bp with the highest frequency in between 907-957 and 957-1007 bp. In Honduras the highest frequency was observed in 700bp group (Hadda *et al.* 1999).

Multiplicity of Infection (MOI)

Mean multiplicity of infection (MOI) of MSP1, MSP2 and GLURP was 1.51, 1.25 and1.08 respectively in overall sample (Table 7.13). Mwingira *et al.* (2011) reported MOI of MSP1, MSP2 and GLURP in several African countries such as Malawi (1.03, 1.52 and 1.01), Burkina Faso (1.4, 3.03 and 1.86), Tanzania (2.50, 3.48 and 1.84) and Uganda (1.18, 1.17 and 1.29). Comparing to those data, a similar MOI for MSP1 and GLURP in *P. falciaprum* Bangladeshi field isolates was observed. However, due to use of smaller bin size (3 bp) Mwingira *et al.* (2011) found high MOI values for MSP2 in their observation. In Papua New Guinea the MOI of MSP1 and MSP2 was 1.99 and 1.84 and for another community in Tanzania that rate was 3.04 and 3.72 respectively (Schoepflin *et al.* 2009). Atroosh *et al.* (2011) reported similar MSP1 (1.37) and MSP2 (1.20) MOI in Malaysia. In Indian state of Assam Baruah *et al.* (2009) reported high MOI values for MSP1 (3.31 to 4.12) but they did not report MSP2 and GLURP in their study. The MOI of MSP1 (2.03) and MSP2 (2.35) found to be higher in Myanmar (Kang *et al.* 2010). Akter *et al.* (2012) reported 2.7 and 1.2 MOI rates for MSP1 and GLURP from Bangladeshi *P. falciparum* sample.

MOI for MSP1 and GLURP in *P. falciaprum* was 1.44, 1.29 and1.11 respectively in non-CHT areas and 1.58, 1.22 and 1.04 respectively in CHT areas in the present observation (Table 7.12). In Pakistan Ghanchi *et al.* (2010) also reported such limited variation in three different regions of Pakistan where they found MOI of MSP1 in Karachi 1.26, Sindh 1.23 and Baluchistan 1.11 respectively. MOI rate for MSP2 was 1.23, 1.25 and 1.00 respectively in their observation.

The expected heterozygosity (H_E)

The expected heterozygosity (H_E) of MSP1, MSP2 and GLURP were 0.80, 0.93 and 0.83 respectively in over all data, 0.84, 0.90 and0.78 respectively non-CHT areas and 0.76, 0.93 and 0.86 respectively in CHT areas (Table 7.12). Mwingira *et al.* (2011) reported H_E of MSP1, MSP2 and GLURP in several African countries such as Malawi (0.79, 0.97 and 0.89), Burkina Faso (0.78, 0.98 and 0.91), Tanzania (0.84, 0.99 and 0.92) and Uganda (0.68, 0.95 and 0.88). In Papua New Guinea the H_E of MSP1 and MSP2 was 0.92 and 0.93 and for another community in Tanzania that rate was 0.92 and 0.97 respectively (Schoepflin *et al*. 2009). Atroosh *et al.* (2011) reported H_E of 0.57 and 0.55 respectively for MSP1 and MSP2 in Malaysia.

Heterozygosity can tell us a great deal about the structure and even history of a population. Higher the heterozygosity higher the genetic variability and lower the heterozygosity lower genetic variability. The value of these measures will range from zero (no heterozygosity) to 1.0 (for a system with a large number of equally frequent alleles). Thus, H_E is > 0.75 means that there is >75% chance of being a heterozygote. In the present study we reveled high level of heterozygosity (0.80-0.93) for all three markers in Bangladesh which is higher than heterozygosity range (0.51-0.65) reported earlier in Southeast Asia/Pacific region but quite comparable to some African countries (0.76-0.80) (Anderson *et al.* 2000). Although, it is believed that in areas where malaria endemcity is declining the heterozygosity of *P. falciparum* genotypes will also decrease (Anderson *et al.* (2000) and Anthony *et al.* (2005) but this is not true for Bangladesh as a declining trend of malaria incidence has been observed in the present study. This is possible that there may be a good number of asymptomatic malaria cases across the country or there may be a large number of unreported malaria cases exist in the endemic population which may eventually contribute in the high genetic diversity as observed in the present study.

The present study found that the frequency of MSP1 allelic families was higher than MSP2 although the number alleles remained higher in MSP2. In the present study almost equal dominance was shown by K1 (63 clones) and MAD20 (64 clones) for MSP1 which is different as seen in Malaysia (Atroosh *et al.* 2011). Earlier dominance of MAD20 was also seen in Thailand (Snounou *et al.* 1999) and Myanmar (Kang *et al.* 2010). However, the dominance of 3D7/IC allelic families in MSP2 seen in the present observation were also common in Malaysia (Atroosh *et al.* 2011), Myanmar (Kang *et al.* 2010) and in sub-Saharan African countries (Mwingira *et al.* 2010).

MSP1, MSP2 and GLURP are three polymorphic region of *P. falciparum* recommended as markers for accessing genotypes in order to study efficacy of anti-malarial drugs. Before the present study genetic variation of these three allelic families were never been studied in different endemic community in the country. The present study could contribute in future to document efficacy of the current anti-malarial drug and investigate new drug or vaccine in Bangladesh.

CHAPTER 8

GENERAL DISCUSSION

GENERAL DISCUSSION

Malaria is a global health problem that caused by five different parasite species of the genus *Plasmodium* in humans (*Plasmodium falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*). *P. falciparum* is the most deadly and predominant than others species. Approximately 3.4 billion people were at risk of malaria globally in 2012 (WHO 2013).

Before the introduction of the Malaria Eradication Program in 1961, the disease was so widespread that it accounted annually about 15 percent of the total deaths in Bangladesh (Robinson 1961). However, successful implementation of the Malaria Eradication Program (MEP) in 1961, the disease was controlled from most of the part of the country but interrupted by the war of Independence in 1917. As a result incidence of malaria in Bangladesh increased again in the years 1972-1977(Paul 1984). However, the MEP was suspended during July 1977 and merged with Malaria and Parasitic Disease Control (M&PDC) Program. During and post MEP period DDT was used as indoor residual spray (IRS) which contributed a rapid reduction of malaria vector and thereby malaria incidence. However, due to its long residual and harmful impact to human health and environment, DDT was banned in 1985 and withdrawn from public health application for such malaria control activity. As a result a resurgence of malaria occurred in 90's.

During the present study period (2010-13), a total of 1,64,055 malaria cases were reported from 13 endemic districts of Bangladesh. Due to support from Global Funds to Fight against AIDS, Tuberculosis and Malaria (GFATM), since 2007 Malaria incidence rate decreased from 6·2 cases per 1000 population in 2008, to 2·1 cases per 1000 population in 2012 (Haque *et al*. 2014). This declining trend was also remain evident in 2013 as the incidence was found 2.0. Thus, malaria is in a declining trend in recent time of Bangladesh.

This trend is also evident in the neighbouring regions. According to current WHO report, the number of confirmed malaria cases reported in the South-East Asia region decreased from 2.9 to 2 million between 2000 and 2012 (WHO 2013).

Available evidence suggests that given equal exposure, adult men and women are equally vulnerable to malaria infection (Reuben 1993). In the present observation we have seen slightly higher malaria incidence rate in male than female. The overall mean female: male ratio of annual malaria incidence was 1:1.42. Historically male are prone to malaria in Bangladesh. Malaria prevalence among male individuals was 1·3 times higher than in female individuals during 2008-12 (Haque *et al.* 2014). However, during first malaria prevalence survey in Bangladesh, the rate of prevalence in female (3.98%) was marginally higher than male (3.96%) (Haque *et al.* 2009a).

Worldwide, between 2000 and 2012, estimated malaria mortality rates decreased by 42% in all age groups and by 48% in children less than 5 years of age. Disease Modeling suggests that an estimated 3.3 million malaria deaths were averted between 2001 and 2012, and that 69% of these lives saved were in the 10 countries with the highest malaria burden in 2000 (WHO 2013). It has been evident that mortality due to malaria has been reduced in a significant rate as indicated in the present study.

In recent time, *P. falciparum* remains the major malaria parasite species in Bangladesh (Haque *et al*. 2009a and Haque *et al.* 2014). This has also been observed in the present study where 94.2% of total malaria cases during 2010-13 were caused by *P. falciparum* alone. *P. vivax* was found to be involved in only 5.5% cases. However, earlier *P. vivax* was the major malaria parasite species in Bangladesh (Paul 1984). The reason could be the emergence of choloroquine resistance *P. falciparum* in Bangladesh in 80's whereas, *P. vivax* still remains sensitive to choloroquine.

During Malaria Eradication Programme (MEP) period Chittagong, the Chittagong Hill Tracts, Sylhet, and Patuakhali were the districts with the highest level of incidence of malaria (Paul 1984). Due to inaccessibility of the Chittagong Hill Tracts, malaria still remains a great public health problem. Topographically, the Chittagong Hill Tracts are the only very hilly area in Bangladesh with stream and forest vegetation. However, due to continuous deforestation of natural vegetation and aforestation with teak and rubber plants a huge number of patchy and isolated malaria hotspots have been established. This kind of hot spots can serve harbour malaria reservoir as malaria vectors does not follow any geographical boundary and can fly in a range from 2-5 km.

In the present observation an increasing trend or a peak of transmission of malaria was seen in between May-October. This data is similar to previous observation Haque *et al.* (2010) in between 1989-2008. This is probably related to rainfall. Except for some small fluctuations, rainfall occurred between May and October most of the year in Bangladesh. In Northern Indian states, malaria infection reports started in April onwards. Rains during June through August trigger increase in malaria-positive cases during September to October (Dutta *et al.* 2010). Singh *et al.* (2003) in Madhya Pradesh, India found more than 70% of the fever cases were actually caused by malaria and of those 87% malaria case was caused by *P. falciparum* in December 1998. During follow up survey, they found 58% of the inhabitants were infected by malaria, with 80% of these cases caused by *P. falciparum* in November 1999. Khan and Talibi (1972) in Khagrachari of Chittagong Hill Tacts observed the transmission of malaria with two peaks, one in the pre-monsoon period, the other in the pre-winter period.

The role of KAP research in the design and implementation of evidence-based prevention, management and control strategies for malaria is very important d (Mwenesi 2005). The potential contribution of KAP studies to malaria research and control has not received much attention in most countries of the world including Bangladesh (Ahmed *et al.* 2009, Hlongwana *et al.* 2009). The present study attempted to address the current knowledge gaps in malaria by presenting data on malaria knowledge, attitude and practices.

It revealed that from the current study, around 65% people in both endemic and non-endemic regions know that malaria is transmitted by mosquito bites but only about 10% people knew that a female anopheline mosquito when had bitten an malaria infected person become infected and can transmit malaria. This is consistent with previous KAP studies in Bangladesh and other countries as observed by Bashar *et al.* (2012) and Ahmed *et al.* (2009).

It was observed in the present study that, people rely mostly on bed nets as a preventive measure against mosquito bite. Similar high level of knowledge on preventive use of bed net was also observed in Bangladesh (Ahmed *et al.* 2009), Nepal (Joshi and Banjara 2008) and Ghana (De La Cruz *et al*. 2006) but lower at

Ethiopia (Jima *et al*. 2005), Iran (Rakhshani *et al.* 2003), Delhi (Tyagi *et al*. 2005) and Turkey (Simsek and Kurcer 2005).

Respondents were familiar with modern allopathic treatment and would accept for treating malaria. Whether formal or informal way they would seek treatment from village doctors or private health centers as revealed from the current study. Unlike to the studies of Turkey (Simsek and Kurcer 2005) self-treatment practice for malaria was not common in Bangladesh.

Current malaria control strategy in Bangladesh mainly relies on the use of ITNs. For several years this strategy has benefited from mass campaign promotions (Ahmed *et al.* 2011). Free distribution of ITNs in other countries also benefitted people from malaria infection and lowered the morbidity (Aderaw 2013). In the current study nearly all the households possessed bed nets and most of them believe bed nets can prevent malaria. Similar situated has also been observed in the previous studies in Bangladesh (Ahmed *et al*. 2009, Bashar *et al*. 2012) and India (Tyagi *et al*. 2005).

Use of insecticide for mosquito control methodology reflects the socio-economic condition of the respondents (Banguero 1984). In the present study, high usage of commercially available mosquito coils by the respondents of non-endemic areas and low in endemic areas explained the impact of socio-economic conditions on the selection of protection means in communities. It has been well established that if mosquito blood meal could be diverted towards wild or domestic animals, which are not the reservoir hosts of malaria, the number of would be reduced (WHO 1982). Recent studies detected *Plasmodium* parasites from some *Anopheles* species which were previously believed to be zoophilic (Alam *et al*. 2010, Alam *et al*. 2012). Thus some of these zoophilic mosquitoes may feed upon humans and could potentially transmit malaria (Alam *et al.* 2012).

The newly developed real-time PCR could detect less than 1 parasite/uL from clinical sample. This is rate similar to previously described real-time PCR method such as Perandin *et al.* (2004), Shokoples (2009) and Tran *et al.* (2014). However, the present study demonstrated the establishment of pre a real-time PCR assay modified with SYBR Green dye for the detection of *P. falciparum* as an alternative. Probe/TaqMan-based real-time PCR is expensive and most of the reference lab in middle and low

income country cannot afford. Instead of probe SYBR Green can be an affordable alternative.

The present study has also established a melting temperature of 74.5 °C for *P. falciparum* from the corresponding positive controls respectively. This will help to distinguish from non targeted amplification (non-falciparum species).

The newly developed real-time PCR assay had 97.1% sensitivity and 97.6% specificity for the detection of *P. falciparum* in clinical isolates. This is a good result compare to acceptable evaluation of new diagnostic method as seen throughout the world (Kamu *et al*. 2007, Mangold *et al.* 2005, Perandin *et al.* 2004). Newly developed real-time PCR provided 97.6 % specificity (95% CI: 94.0%-99.3%) which is also a comparable result globally. In terms of specificity, Rantala *et al.* (2010) reported 91.2% with their real-time PCR method for *P. falciparum*. However, Perandin *et al.* (2004) reported 100%.specificty with the developed real-time PCR method for the detection of *P. falciparum*

The LAMP primer sets assessed in the present study were able to detect clinical *falciparum* malaria with great accuracy in comparison with microscopy and real-time PCR. LAMP with newly designed primers is more sensitive as it can detect a minimum of five *P. falciparum* parasites/µL of human blood. But with the old primer set Poon *et al.* (2006) the detection limit we obtained 100 copies from clinical samples.

Since this primer set can detect five parasites/µL, it required approximately 15 copies of template to obtain significant visible amplification. However, Notomi *et al*. (2000), in their initial design mentioned that LAMP method can conduct visible amplification from six copies of template within 45 minutes when primer designing and reaction conditions are perfectly optimized.

Poon *et al.* (2006) described 95% sensitivity and 99% specificity in regard to PCR in their first implementation of LAMP in malaria. The same primer set was replicated in the genus-species specific LAMP described by Han *et al.* (2007), where all of the 12 microscopy and PCR positive *P. falciparum* samples were successfully detected by LAMP. Poschl *et al.* (2010) described 100% sensitivity and specificity of LAMP compared to the nested PCR using the same primer set and reaction conditions.

Lucchi *et al*. (2010) have also utilized the same primer set in real-time fluorescence LAMP with portable fluorescence reader. That study found 98.8% sensitivity with 100% specificity of the method while Paris *et al.* (2007) denoted 73.1% sensitivity compared to microscopy using same primer set with specificity 100%.

Yamamura *et al.* (2009) designed another 18S rRNA based primer set for *P. falciparum* detection, which can detect 10 copies of plasmid DNA after 80 minutes of amplification. This method was determined to be as 97.8% sensitive and specificity was found to be 85.7%. However, cross reactions of the primers with *P. vivax* and other undefined targets was ignored by melt curve analysis. This method also required sophisticated instruments like Genopattern Analyzer GP1000 which limits wide application of their method.

Mitochondrial DNA has 20 to 150 copies in the same parasite. Polley *et al.* (2010) described another primer set targeting mitochondrial DNA for detection of *P. falciparum* malaria. Thus this set also can detect 5 parasites/µL. This primer set is 93.3% sensitive and 100% specific to identify *P. falciparum* cases. However, the LAMP with newly designed primers was found 99.1% (95% CI, 94.9-100) and 98.1% (95CI%, 93.5-98.8) sensitive compared to microscopy and real-time PCR, respectively while specificity was 99% (95% CI, 94.8-100) and 100% (95% CI, 96.5-100) for the assessment of *P. falciparum* clinical isolates reveal a good promise for future use in the field level molecular diagnosis in resource limited settings.

A total of 168 MSP1 clones belonging to 14 alleles were found in Bangladesh by MSP1 specific PCR in three polymorphic regions. In some African countries the number of MSP1 alleles were: 16 in Tanzania, 13 in Malawi , 10 in Uganda, 15 in Burkina Faso (Mwingira *et al.* 2011) and 33 in Senegal (Konate *et al.* 1999). In an observation, a total of 14 different alleles of MSP 1 were recognized in Myanmer (Kang *et al.* 2010). Due to monomorphic nature frequency of RO33 allelic family was found highest (31.5%) in overall distribution. In K1allelic family the highest contribution (13.1%) for frequency distribution was reported in 201-225 bp categories from overall data. Whereas, the contribution of on K1 frequency distribution reported from Non-CHT in 201-225 base pair group (7.7%) and from CHT in 226-250 base pair group (6.2%) respectively. In MAD allelic family the highest contribution (16.9%) for frequency distribution was reported in 176-200 bp categories from overall

data. Whereas, the contribution of on K1 frequency distribution reported from Non-CHT in176-200 base pair group (6.9%) and 10% and from CHT in 151-175 and 176-200 base pair groups (10%) respectively (Figure 7.12).

In the present observation, a total of 128 clones belonging 20 alleles were found by MSP2 specific PCR in two polymorphic regions (FC27 and 3D7). Mwingira *et al.*(2011) adopted a smaller bin size of 3 bp and thus got high number MSP2 alleles in some African countries such as: 73 in Tanzania, 69 in Malawi , 49 in Uganda, 97 in Burkina Faso (Mwingire *et al.* 2011). In the same method, Schoepflin *et al*. (2009) found 35 alleles in Papua New Guinea and 76 alleles in Tanzania and 33 in Senegal (Konate *et al.* 1999). However, by using the same methodology like the presenr study, 12 alleles reported by Paul *et al*. (1998) from Thailand and 19 alleles from Gabon by Aubouy *et al.* (2003).

A total of 141clones belonging to 13 allelic families were found by GLURP specific PCR in the present study. Mwingire *et al.* (2011) reported 14 alleles in Burkina Faso, 13 alleles both in Malawi and Tanzania and 10 alleles from Uganda. Only two alleles of GLURP were reported in Honduras by Hadda *et al.* (1999).

Mean multiplicity of infection (MOI) of MSP1, MSP2 and GLURP was 1.51, 1.25 and1.08 respectively in overall samples 1.44, 1.29 and1.11 respectively in non-CHT areas and 1.58, 1.22 and 1.04 respectively in CHT areas (Table 7.13).
Mwingira *et al.* (2011) reported MOI of MSP1, MSP2 and GLURP in several African countries such as Malawi (1.03, 1.52 and 1.01), Burkina Faso (1.4, 3.03 and 1.86), Tanzania (2.50, 3.48 and 1.84) and Uganda (1.18, 1.17 and 1.29). Comparing to those data, a similar MOI for MSP1 and GLURP in *P. falciaprum* Bangladeshi field isolates was observed. In Pakistan Ghanchi *et al.* (2010) also reported such limited variation in three different regions of Pakistan where they found MOI of MSP1 in Karachi 1.26, Sindh 1.23 and Baluchistan 1.11 respectively. MOI rate for MSP2 was 1.23, 1.25 and 1.00 respectively in their observation.
The expected heterozygosity (H_E) of MSP1, MSP2 and GLURP were of MSP1, MSP2 and GLURP in over all data 0.80, 0.93 and 0.83 respectively, was found 0.84, 0.90 and0.78 respectively in Non-CHT areas and 0.76, 0.93 and 0.86 respectively in CHT areas (Table 7.13). Mwingira *et al.* (2011) reported H_E of MSP1, MSP2 and

GLURP in several African countries such as Malawi (0.79, 0.97 and 0.89), Burkina Faso (0.78, 0.98 and 0.91), Tanzania (0.84, 0.99 and 0.92) and Uganda (0.68, 0.95 and 0.88). In Papua New Guinea the H_E of MSP1 and MSP2 was 0.92 and 0.93 and for another community in Tanzania that rate was 0.92 and 0.97 respectively (Schoepflin *et al*. 2009). Atroosh *et al.* (2011) reported H_E of 0.57 and 0.55 respectively for MSP1 and MSP2 in Malaysia.

In the present study we reveled high level of heterozygosity (0.80-0.93) for all three markers in Bangladesh which is higher than heterozygosity range (0.51-0.65) reported earlier in Southeast Asia/Pacific region but quite comparable to some African countries (0.76-0.80) (Anderson *et al.* 2000). Although, it is believed that in areas where malaria endemcity is declining the heterozygosity of *P. falciparum* genotypes will also decrease (Anderson *et al.* (2000) and Anthony *et al.* (2005) but this is not true for Bangladesh as a declining trend of malaria incidence has been observed in the present study. This is possible that there may be a good number of asymptomatic malaria cases across the country or there may be a large number of unreported malaria cases exist in the endemic population which may eventually contribute in the high genetic diversity as observed in the present study.

The present study found that the frequency of MSP1 allelic families was higher than MSP2 although the number alleles remained higher in MSP2. In the present study almost equal dominance was shown by K1 (63 clones) and MAD20 (64 clones) for MSP1 which is different as seen in Malaysia (Atroosh *et al.* 2011). Earlier dominance of MAD20 was also seen in Thailand (Snounou *et al.* 1999) and Myanmar (Kang *et al.* 2010). However, the dominance of 3D7/IC allelic families in MSP2 seen in the present observation were also common in Malaysia (Atroosh *et al.* 2011), Myanmar (Kang *et al.* 2010) and in sub-Saharan African countries (Mwingira *et al.* 2010).

MSP1, MSP2 and GLURP are three polymorphic region of *P. falciparum* recommended as markers for accessing genotypes in order to study efficacy of anti-malarial drugs. Before the present study genetic variation of these three allelic families were never been studied in different endemic community in the country. The present study could contribute in future to document efficacy of the current anti-malarial drug and investigate new drug or vaccine in Bangladesh.

CHAPTER 9

SUMMARY

SUMMARY

The present study attempted to link between basic malaria epidemiology to development of high level molecular diagnostic methods and finally looked at the genetical variations of *Plasmodium falciparum*, the most virulent malaria parasite in Bangladesh and in the world.

Observation on Epidemiology of malaria in Bangladesh

In the present study different aspects of malaria epidemiology in Bangladesh were studied. Epidemiological data on malaria during 2010-2013, from 13 endemic districts were collected on a monthly basis. Available data included the number of persons diagnosed as malaria patients by either microscopy or by Rapid Diagnostic Test (RDT), age, sex, type of infection etc. All collected data were checked for its reliability and consistency with the National Malaria Control Programme (NMCP) and analyzed.

Annual Malaria Incidences

During the study period (2010-13), a total of 1,64,055 malaria cases were reported from 13 endemic districts of Bangladesh. Highest number of cases 55,873 was reported in the year 2010 followed by 51,773 in 2011, 29,518 in 2012 and 26,891 in 2013 respectively.

Prevalence of malaria according to sex and age groups

During the study period (2010-13), a total of 96,189 male and 67,866 female were identified as malaria patients. The number of male vs. female infection was 31,669 and 24,024 in 2010; 30,531 and 21,242 in 2011; 17,621 and 11,897 in 2012 and 16,368 and 10,523 in 2013 respectively. Male are more prone to malaria infection than female. The overall mean female: male ratio of annual malaria incidence was1:1.42. However this rate was 1:1.31 in 2010; 1: 1.44 in 2011; 1:1.48 in 2012 and 1:1.56 in 2013 respectively.

According to the age group, highest malaria incidence was always observed in working age group 15+ years. A total of 100,413 (%) malaria cases were reported from this age category. This number was followed by 44,823 (%) in 5-14 years of age

group, 17,628 (%) in 1-4 years of age group and 1,191 in less than one year age group respectively.

Malaria caused by *Plasmodium falciparum* was found dominant in Bangladesh. A total of 154,562 (94.2%) *P. falciparum* infected cases were detected (Figure 3.4). Only 5.5% cases were caused by *P. vivax*. Remaining infection was caused by *P. falciparum* and *P. vivax* mixed infection.

Malaria in Pregnancy

During the study period a total of 497 malaria cases were identified among pregnant women in endemic areas of Bangladesh. Highest number of pregnant women (153) was affected in the year 2011 and lowest (106) in 2013. *P. falciparum* remains the main causing agent for malaria during pregnancy. The highest number of *P. falciparum* infection among pregnant women was 140 in 2011 and lowest 106 in 2013. The prevalence of *P. falciparum* infecting women ranges between 88.6%-91%. The highest number of *P. vivax* infection among pregnant women was 14 in 2010 and lowest 0 in 2013. Overall contribution of *P. vivax* infected malaria among pregnant women ranges from 9.3%-12.8% during this period.

Death due to malaria

A total of 99 patients died during the study period (2010-13). Quiet opposite of the malaria incidence, female (51) showed slightly higher death than male (48). Highest number of death was 37 in 2010 followed by 36 in 2011 (Table 3.2). Lowest number of death due to malaria was 11 in 2012 followed by 15 in 2013. Thus, case fatality rate during 2010-13 was 7.1, 7.3, 4.0 and 5.8 respectively per 10,000 *P. falciparum* malaria infected person that also include mixed infection (Table 3.2 and Figure: 3.9). However, for female this rate became 8.0; 10.8; 3.5 and 6.8 respectively from 2010-13. Male only had high mortality rate over female in 2012. During remaining year (2010- 11 and 2013), female had higher fatality rate due to severe malaria (Figure: 3.9).

Malaria according to districts:

Bandarban district recorded the highest malaria burden in Bangladesh. The numbers of cases in all four years were 17,259 in 2010, 16,097, 8,461 and 9,459 in 2011, 2012, and 2013 respectively. Rangamati district remains second position throughout this

period with 13,949; 13,669; 7,981 and 7,976 respectively during 2010-13. Khagrachhari district remain the third position with 12,348; 12,952; 5,997 and 4,096 cases respectively during 2010-13 (Table 3.2 and Figure 3.10).

Apart from these three districts of Chittagong Hill Tracts (CHT) significant number of malaria cases were reported in Cox's Bazar district with 6,414; 5,472; 3,888 and 3,252 cases respectively followed by Chittagong with 1,624; 1,415; 1,095 and 648 cases respectively from 2010-13 (Table 3.2 and Figure 3.10).

In Sylhet zone highest number of cases were reported from Sunamgonj district with 1,134; 461; 540 and 488 cases respectively from 2010-13, followed by Sylhet district (882; 444;436 and 360 respectively (Table 3.2 and Figure 3.10).

In northern zone, the highest numbers of cases were reported from Netrokona district with 375; 270; 285 and 199 cases respectively followed by Mymensing district with 418; 244; 168 and 74 malaria cases respectively during 2010-13 (Table 3.2 and Figure 3.10).

In Chittagong Hill Tracts districts (Bandarban, Rangamati and Khagrachari) accounted for almost 80% of total malaria cases of the country. Precisely Chittagong hill tracts contributed 78% of country's total malaria cases in 2010; 82.5% in 2011; 76% in 2012 and 80.1% in 2013 respectively (Figure 3.11). Bandarban of Chittagong hill tracts region contributed on average 31.3% cases reported in between 2010-13. This contribution remains 30.9%, 31.1%, 28.7% and 35.2% respectively during 2010-13 (Figure 3.12 to 3.15).

Prevalence of *Plasmodium* species according to districts

Throughout the study period, Bandarban remains as the highest *P. falciparum* burdened district in Bangladesh. A total of 49,389 (31.9%) *P. falciparum* infected malaria cases were identified in Bandarban during the period 2010-13. Bandarban also recorded highest *P. falciparum* infected malaria cases in all four years (2010-13). The second most infected district for *P. falciparum* infection was Rangamati (41,396; 26.8%) followed by Khagrachari (34,469; 22.2%). Outside of the Chittagong hill tracts, Cox's Bazar represented the highest number of *P. falciparum* cases (17,398; 11.3%) during the study period (Table 3.3 and Figure 3.16).

Throughout the study period, Rangamati district remains as the highest *P. vivax* burdened district in Bangladesh. Out of 9,043 *P. vivax* infected cases in Bangladesh, 2,092 cases were identified in Rangamati district during 2010-13. . This number accounted for 23.1% total *P. vivax* infection in the country. The second most infected district for *P. vivax* infection was Bandarban (1,671; 18.5%) followed by Cox's Bazar (1,621; 17.9%). Rangamati recorded highest *P. vivax* infected malaria cases in 2010 and 2011 but Cox's Bazar was observed to be highest in 2012 and 2013 (Table 3.4 and Figure 3.17).

According to *Plasmodium* species, all endemic districts except for Kurigram showed higher number of *P. falciparum* infection than *P. vivax* infection. However, Khagrachari recorded the highest percentage (97.4%) of *P. falciparum* infection among total case reported during 2010-12. On the other hand Kurigram reported the highest percentage (72.6%) of *P. vivax* infection among total case report during 2010-13(Figure 3.18). Among the total case reported during 2010-13, no district was found to have equal representation of these two malaria parasite species except for Sherpur which has 2: 1 ratio represented by 67.8% of *P. falciparum* infection over 32.2% *P. vivax* infection among total case reported during 2010-13 (Figure 3.18).

Temporal distribution of Malaria

Malaria has been observed to be prevalent throughout the year in Bangladesh. However, an increasing trend or a peak of transmission was found in between May-October. More precisely, malaria incidence was found highest during June- July of a particular year. During 2010 -2011, June was the most prevalent month (7554 and 8478 respectively) while July was the most malaria prevalent month in 2012 and 2013 (4333 and 5299 respectively). Overall, in July 15.4% and June 14.3% of country's total malaria cases were recorded. The lowest monthly incidence was recorded in March (4.4%) followed by February (4.7%) during 2010-13 (Table 3.6 and Figure 3.19). An overall monthly malaria trend of malaria in recent years is presented in Figure 3.20.

Overall trend of malaria

A decreased overall trend for malaria transmission has been during 2010-13 from previous years. In Bangladesh from 2010-2009, on average annual malaria incidence

was 57,587 whereas during 2010-13 the average annual cases incidence was 41,014. The average annual malaria incidence for most recent four years (2006-2009) was 60,319. Thus, in all aspect in recent years a decreasing trend for malaria incidence is evident in Bangladesh.

Overall malaria has a declining trend in Bangladesh in recent years which is also evident in the present observation. Since 2007, Global Funds to Fight AIDS, Tuberculosis and Malaria (GFATM) since 2007 Malaria incidence rate decreased significantly. Our national malaria control programme (NMCP) adopted early diagnosis by providing Rapid diagnostic test (RDT) to the community level, provided the best available treatment regimen (artimisinin based combination therapy) and vector control through insecticide treated bed-net (ITN) and Long lasting insecticide treated bed-net (LLIN). As a collateral effect of these malaria incidence and case fatality due to malaria has been decreased significantly.

However, the alarming fact is that the present high abundance of *P. falciparum* (>90%) which needs to be bring down in below threshold level. This species (*P. falciparum*) is the most virulent that can cause significant mortality and morbidity. This parasite also has ability to develop resistance to antimalaria drugs. Thus in this is very important to monitoring of efficacy of existing drugs against *P. falciparum* in a regular basis.

KAP regarding malaria in Bangladesh

In the current study, a total of 660 individuals (110 from each area) from three malaria endemic (Khagrachari, Sylhet and Cox's Bazar) and three non-endemic districts (Dhaka, Rajshahi and Bagerhat) of Bangladesh were selected for KAP study. The survey was conducted in portions of six different malaria endemic and non-endemic areas of Bangladesh from May 2012 to July 2014. A standard structured questionnaire was designed to collect information irrespective of sex, religion and ethnicity. Interview was taken from a respondent of each household who is above or at 10 years of age.

Socio-Demographic Characteristics of the Respondents

Majority of the respondents were female (58%) where most of the respondents (29.7%) lies in the age group of 30-39 years in non-endemic area and more than 26%

people in the age group of 20-29 in the endemic area. Family size was significantly smaller in non-endemic areas than endemic areas where more than 60% households possess 5 - 8 members (Table 4. 2). In both areas, most of the respondents were married (> 82%) and deprived of any formal education (> 30% and > 50% in non-endemic and endemic region respectively). In the non-endemic area, housework became the most common occupation (30%). On the other hand, daily labor was the common (34%) means of earning livelihood in the endemic area (Table 4. 2).

Knowledge on malaria and awareness

The term 'Malaria' was well known to the community. More than 50% respondents of endemic area reported that they had experienced malaria among the household members and 24.5% of them said that there was at least one malaria patients in their house within a year. Number of infections in the past five years between non-endemic and endemic areas was significantly different ($p < 0.05$) (Table 4. 3).

Mosquito bite is responsible for malaria was known to more than 60% of the respondents from both areas. Where malaria transmission was not well known in the non-endemic area but 66% respondents of endemic area reported that people get malaria by mosquito bite. However, the cause of malaria transmission that a female infected anopheline transmits the disease remained vague in both areas (Table 4. 3). More than 70% of the respondents didn't know the symptoms of malaria in the non-endemic area whereas; 'onset of fever with shivering' was the common answer among the respondents of the endemic area. Significant ($p < 0.05$) difference was observed among the responses of two areas regarding the symptom of malaria (Table 4. 3). In both areas, people seemed to rely mostly on bed nets (more than 40%) to prevent malaria and if they become infected, allopathic medicine was the choice of treatment (about 90%).

The treatment seeking behavior varied significantly ($p < 0.05$) between the respondents of the two areas. Government hospitals were chosen by most (41%) of the inhabitants of non-endemic area whereas, private health center (> 30%) and village doctor (> 30%) were preferred by the respondents of endemic area. Non-government health workers seemed to play key role of circulating malaria awareness in endemic area (Table 4. 3).

Use and possession of bed nets

Almost all the respondents were explicitly reported to have at least one bed net (> 98%) despite a tiny fraction who did not have any bed net. In the endemic area nearly 80% of the respondents reported that they use bed nets regularly and most of the respondents believed that use of bed nets can prevent malaria. However, still a high percentage of respondents (more than 50%) contracted malaria.

Household economic characteristics of the respondents' and practices

Most of the households were observed to be 'Not Jhupri' i.e. permanent/semi permanent structure. Construction of household partition mostly done by mud (51%) in endemic areas which is significantly ($p < 0.05$) different from households in non-endemic areas where household partitions were mostly cemented (55%). Mud floor was also common is endemic areas (84%), where in non-endemic areas mud and cemented floors are both equally popular. Significant ($p < 0.05$) difference in terms of economic status has been observed between non-endemic and endemic areas. Nearly 50% of the respondents from non-endemic areas reported that their income is surplus, on the other hand more than 30% respondents from endemic areas reported that their monthly income is sometime deficient (Table 4. 6). Although most respondents from both areas reported that their economic condition has not been improved within the past five years, respondents of the endemic areas seemed to pass more deteriorated economic change in last five years (Table 4. 6).

Estimation of relative risk factors for malaria in Bangladesh

Transmission of malaria has been observed to be significantly ($p < 0.05$) related with the educational status. Respondents who are graduate or equal educational qualification had two times more knowledge on the malaria transmission related information compare to people who had no formal education (Table 4. 7). People of the endemic areas who had completed master education knew 1.3 times more about the cause of malaria than people of the non-endemic area (Table 4. 8). On the other hand, people who undertook graduation was observed to know 1.5 times more about the malaria symptoms (Table 4. 9). Knowledge on the malaria transmission has been possessed more than 2 times by the families who earn more than BDT 20000 comparing with those families who earn BDT 5000 (Table 4. 10). Use of insecticides for mosquito control has observed to be significantly related with the monthly income

of the family. Families whose monthly income exceeds BDT 20000 per month use insecticides 3 times more than the base group (Table 4. 11). Likewise, possession of bed nets has observed to be strongly associated with the wealth of the family. More bed nets have also been significantly possessed by the families of more monthly income (Table 4. 12). However, families whose income limited to BDT 5000 and > BDT 5000 – 10000 significantly answered that they had malaria patients in the past 5 years, which was more than 2 and 3 times respectively than families earning > BDT 20000 (Table 4. 13).

Comparison of general practices and knowledge among the respondents between non-endemic and endemic areas

Equal number of respondents reported to reside outside of the house at the evening time. However, market places and tea stalls seemed to be the popular places to spend time for the respondents of endemic area (Figure 4. 5). Although equal number of respondents knew 'mosquito bite' is responsible for malaria (about 65%) (Figure 4. 6) but number of malaria cases in past 5 years were significantly common in endemic areas among the respondents (> 50%) (Figure 4. 7). Where the knowledge 'bitten by any mosquito' can cause malaria was significantly common among the respondents of endemic area than non-endemic area, the actual cause of transmission that 'bitten by an infected mosquito' was known to few respondents (Figure 4. 8). Regarding the symptom of malaria, respondents of the endemic areas significantly answered correctly than the respondents of the non-endemic area that onset of fever with rigor is the common symptom malaria (about 70%) (Figure 4. 9). Interestingly, use of bed net to prevent malaria answered by more responded of the endemic area than non-endemic area. This might be the cause of the distribution of ITNs among them (Figure 4. 10). Allopathic treatment seemed to be the most common (> 86%) means of treatment in both areas where the respondents of non-endemic areas preferred to go to government hospitals but private health center and village doctor were equally popular (33.6% for both answer) for the inhabitants of endemic area (Figure 4. 11). A significant relationship has been observed between the use of insecticide and economic status. Respondents of the non-endemic areas reported to use more mosquito coil (> 52%) than the respondents of the endemic areas (Figure 4. 12). The most common source of malaria information was observed to be obtained from non government health workers in the endemic areas (Figure 4. 13).

Establishment and evaluation of Real-time PCR for *Plasmodium falciparum*

In the present study a total of 338 febrile patients were enrolled from the Matiranga Upazila Health Complex (UHC) from July 2010-May 2012. Five ml of blood was taken from an adult subject and in case of children or minor subjects three ml of blood was obtained through veni-puncture. Two drops of sample were used for preparing thick and thin smear slides, one drop was used for Paracheck RDT, and the remaining samples were preserved in an EDTA tube and stored at -20°C. DNA was extracted from 200 µl EDTA preserved blood samples using the QiaAmp blood mini kit (QIAGEN, Inc., Germany) following the manufacturer's instructions at the Parasitology Laboratory of icddr,b. DNA sample was stored at 4 °C until PCR could be completed.

Analytical sensitivity Real-Time PCR

In the present investigation, after having the successful amplification by real-time PCR method detection limit (analytical sensitivity) was established following the typical amplification curve. Typical displays (amplification plots) for *P. falciparum* by the SYBR Green I PCR assay provided by Bio Rad CFX-96 are shown in Figures 5.4. Positive signals by means of cycle threshold [CT] value were obtained for all dilutions, with a detection limit of 5-10 parasites/µl for *P. falciparum* in different experiments. The new real-time PCR could detect less than 1 parasite/uL from clinical sample.

Melt curve analysis

The melting peak for *P. falciparum* was found at 74.5 °C from the corresponding positive controls respectively. Any amplification other than these two melting temperatures was excluded as false amplification.

Evaluation of Real-Time PCR

Using the real-time PCR assay results 170 (50.3%) samples were found positive for any malarial infection (Table 5.1). Sensitivity, specificity, positive predictive value, negative predictive value, and kappa (k) of PCR assay compared to microscopy are given in table 5.2. For the detection of *P. falciparum* (including mixed infection), modified real-time PCR assay had 97.1% (95% CI: 93.3-99) sensitivity and 97.6% (95% CI: 94-99.3) specificity respectively (Table 5.2).

Real-time PCR vs. Parasite count

According to parasite count (per uL) by microscopy the newly established real-time PCR provided good result. Based on parasite count 7 categories were made: 1-100/ uL, 101-200/ uL, 201-500/ uL, 501-1,000/ uL, 1,001-5,000/ uL, 5001-10,000/ uL and >10,000/ uL. Among the positive samples 118 were in the >10,000/ uL category and real-time PCR could peal all of those, thus success rate was 100%.

Sensitivity according to parasite count

Sensitivity of the newly developed real-time PCR method has been calculated according to different parasite count. Except for the count group 101-200 uL which had 42.9% sensitivity rate (95% CI, 11.8-%-78.8%), all six categories were 100% sensitive (95% CI 19.8%-100%) (Table 5.4). Due to small number of samples (N=5 to 17) in five categories 1-100/ uL, 201-500/ uL, 501-1,000/ uL, 1,001-5,000/ uL and 5001-10,000/ uL the lower limit was within 78%.

Establishment and Improvement of Loop Mediated Isothermal Amplification (LAMP) for *Plasmodium falciparum* diagnosis

LAMP is a single tube technique for the amplification of DNA (Notomi *et al.* 2000). This method may be useful in future as a low cost alternative to detect certain diseases. Out of 338 samples obtained from the Matiranga Health Complex 106 microscope-positive *Plasmodium falciparum* mono-infection and 105 microscopically negative samples were included in the present study. Thus, a total of 211 samples were taken in to the LAMP experiment. In addition a negative panel consists of 43 DNA samples of *P. vivax*, *P. malariae. Leishmania donovani* and tuberculosis were also tested to check analytical specificity of the LAMP methods.

Initially LAMP method was carried out following the protocol and primer set described by Poon *et al.* (2006), although pre-addition of 120µM HNB was introduced in the reaction mixture. 2 µL of extracted DNA was used as template for amplification. The tubes were heated at 60˚C for 60 minutes in a heat block. Later LAMP experiment was performed with a new primer set designed for the present study.

Limit of detection of old vs. new LAMP

The old LAMP primers by Poon *et al.* (2006) detected a minimum of100 parasites/µL within 60 minutes. This experiment was repeated five times with same end results. No alteration in the initial color was observed after heating the samples up to 90 minutes. Conversely, LAMP with newly designed primers successfully detected 5 parasites/µL within 35 minutes. This experiment was repeated five times with same end results. Out of the five repeated experiments, additional heating to 60 min resulted detection of one parasite/µL in two repeated amplifications.

All the 43 samples in negative panel of *P. vivax*, *P. malariae, Leishmania donovani* and tuberculosis tested negative by both of the LAMP primer sets. Thus analytical specificity with this panel was 100% for both primer sets.

Detection against Microscopy and real-time PCR

Out of 211 DNA samples, 106 were positive in microscopy and 108 by real-time PCR. Out of 106 microscopy-positive samples old LAMP could detect 102 whereas, new LAMP could detect 105. On the other hand, out of 108 real-time PCR positive samples old LAMP could detect 103 but the new LAMP detected all 106. Out of 105 negative samples 2 samples were gave positive response in old LAMP but only 1 by the new LAMP method (Table 6.4).

Sensitivity and specificity of LAMP

Old LAMP method was 96.2% (95% CI, 90.06-99.0) sensitive and 98.1% (95% CI, 93.3-99.8) specific in comparison with microscopy. Meanwhile, sensitivity decreased slightly to 95.4% (95% CI, 88.4-98.0) with somewhat improved specificity when real-time PCR was considered as the reference method (Table 6.5). LAMP with newly designed primers was 99.1% (95%CI, 94.9-100) and 98.1% (95CI%, 93.5-98.8) sensitive compared to microscopy and real-time PCR respectively while specificity was 99% (95% CI, 94.8-100) and 100% (95% CI, 96.5-100) for the assessment of *P. falciparum* DNA samples (Table 6.5).

Genetic diversity of *Plasmodium falciparum*

The study samples were collected from 7 malaria endemic districts (Bandarban, Rangamati, Khagrachari; Netrokona, Mymensingh, Sylhet and Cox's Bazar). Out of these 7 districts 3 were from Chittagong Hill Tracts (CHT) region (Bandarban,

Rangamat and Khagrachari), and 4 were from other endemic areas (non-CHT) (Netrokona, Mymensingh, Sylhet and Cox's Bazar). Febrile patients referred to microscopy for malaria diagnosis of several Upazila Health Complexes of those seven selected districts from July 2010-May 2013 followed by confirmation of *Plasmodium falciparum* mono infection by microscopy, nested PCR and/or real-time PCR were enrolled. DNA was extracted from 200µL preserved whole blood using QiaAmp blood mini kit (Qiagen GmbH, Germany) following manufacturer's instructions. PCR amplification of template DNA and analysis of region II of GLURP, central polymorphic region of MSP2 (3D7/IC and Fc27 allelic families), and block 2 of MSP1 (K1, MAD20 and RO33 allelic families) was performed followed by the method described by the Snounou *et al.* (1999).

MSP1 PCR and clones

Out of 130 samples, 111 were positive for MSP1 PCR (85.4%). In CHT areas, 59 out of 69 samples (85.5%) were amplified and in non-CHT areas, 52 out of 61 samples (85.2%) were amplified respectively in MSP1 specific PCR (Table 7.4). A total of 168 MSP clones were found by MSP1 specific PCR of which 93 (55.4%) were of CHT areas and remaining 75 were of (44.6%) from non-CHT areas. Thus, number of MSP1 clone per positive samples in CHT was 1.57 in CHT areas and 1.44 in non-CHT areas.

MSP1 genotypes per allelic-family/locus

A total 14 alleles were found in Bangladesh by MSP1 specific PCR in three polymorphic regions (K1, MAD20 and RO33). An equal number of 13 allelic families were found in both CHT and non-CHT areas (Table 7.5). However, most of the alleles were common for both the areas.

K1 allele family of MSP1

Out of 63 clones, 7 allele groups of k1 allelic family were recognized from total study samples during the present study (Table 7.5). Of which 6 were common in both the areas. An additional allele from k1 family was found in CHT area. Molecular size varies from 126-300 base pairs in overall and CHT samples and 150-300 base pairs in non-CHT samples (Table 7.6). A typical gel image of K1 family is given in Figure 7.3. According to frequency in Non-CHT areas, the highest number (10, 15.9%) of clones was found in 201-225 base pair region. On the other hand in CHT areas, the

highest number (8, 15.9%) of clones was found in 226-250 base pair region. In overall sample, the highest number (17, 27%) of clones was found in 201-225 base pair region (Figure 7.4-7.5).

MAD20 allele family of MSP1

Out of 64 clones of MAD20 allelic family, 6 allele groups were recognized from total study samples during the present study (Table 7.5). Of which 5 were common in both the areas. An additional allele from MAD20 family was found in CHT area. Molecular size varies from 126-325 base pairs in overall and Non-CHT samples and 126-250 base pairs in non-CHT samples. In non-CHT area the highest number of clones (9,14.1%) was found in 176-200 base pair region. Whereas, in CHT areas the highest number of clones (13, 20.3%) was found in two size groups: 151-175 and 176-200 base pair region (Table 7.7). In overall sample the highest number of clones (22, 34.4%) was found in 176-200 base pair region followed by 151-176 base pair group where (21, 32.8%) samples were obtained (Figure 7.6 and 7.7).

RO33 allele family of MSP1

RO33 family is considered as monomorphic. Therefore, out of 41 clones only 1 allele group of RO33 allelic family was recognized from total study samples during the present study (Table 7.5). The exact molecular size of RO33 was 160 bp and it was found in 16 isolates in non-CHT areas and 25 isolates in CHT areas respectively (Figure 7.10 and 7.11).

Over all frequency of MSP1 allelic group

Being monomorphic clones of RO33 allelic family was found highest (31.5%) in overall distribution. However, the contribution of non-CHT and CHT areas on RO33 frequency distribution were 12.3% and 19.2%, respectively. In K1allelic family, the highest frequency distribution (13.1%) was reported in 201-225 bp categories from overall data. Whereas, the contribution of on k1 frequency distribution reported from non-CHT in 201-225 bp group (7.7%) and from CHT in 226-250 bp group (6.2%), respectively. In MAD20 allelic family, the highest contribution (16.9%) for frequency distribution was reported in 176-200 bp categories from overall data. Whereas, the contribution of on MAD20 frequency distribution reported from non-CHT in176-200 bp group (6.9%) and from CHT in 151-175 and 176-200 bp groups (10% in each), respectively (Figure 7.12).

MSP2 PCR and clones

Out of 130 samples, 102 were positive for MSP2 PCR (78.5%). In CHT areas, 51 out of 69 samples (73.9%) and in non-CHT areas, 51 out of 61 samples (83.6%) contained MSP2 clones (Table 7.8). A total of 128 clones were found by MSP2 specific PCR of which 66 (51.6%) were from non-CHT areas and remaining 62 (48.4%) were from CHT areas (Table 7.8). Thus, number of MSP2 clone per positive samples was 1.29 in non-CHT areas and 1.22 in CHT areas.

MSP2 genotypes per allelic-family/locus

A total 20 alleles were found in Bangladesh by MSP2 specific PCR in two polymorphic regions (Fc27 and 3D7). Number of allelic families was not equal in both areas. In Non-CHT area 14 alleles were found whereas, in CHT areas 16 alleles were found (Table 7.9). However, most of the alleles were common for both the areas.

Fc27 allele family of MSP2

Out of 54 clones, 8 allele groups of Fc27 allelic family were recognized from total study samples during the present study (Table 7.8). Of which 4 were common in both the areas. Molecular size varies from 251-475 base pairs in overall and non-CHT samples and 251-425 base pairs in CHT samples. According to frequency, in non-CHT area the highest number (11, 20.4%) was found in 276-300 base pair region. On the other hand in CHT areas the highest number (11, 20.4%) of clones w found in 301-325 base pair region. In overall sample, the highest number (17, 31.5%) of clones was found in 276-300 base pair region (Figure 7.14-7.15).

3D7/IC allele family of MSP2

Out of 74 clones, 12 allele groups of 3D7/IC allelic family were recognized from total study samples during the present study. Each area had 9 alleles of which 6 were common in both the areas (Table7.9). Molecular size varies from 351-675 base pairs in overall samples, 376-675 in Non-CHT and 351-625 base pairs in CHT samples respectively. According to frequency in Non-CHT area the highest number (11; 14.9%) of clones was found in 476-500 base pair region (Table 7.11). Whereas, in CHT areas the highest number of clones (11; 14.9%) was found in 501-525 base pair region. In overall sample the highest number of clones (17, 23%) was found in 501-525 base pair region (Figure 7.17-7.18).

Over all frequency of MSP2 allelic group

In Fc27allelic family the highest contribution (13.1%) in frequency distribution was reported in 276-300 bp category from overall data. The highest contribution of Fc27 frequency distribution reported from Non-CHT was also in 276-300 bp group (8.5%) and from CHT in 301-350 bp group (8.5%) respectively. In 3D7/IC allelic family the highest contribution (13.1%) for frequency distribution was reported in 501-525 bp categories from overall data. Whereas, the highest frequency distribution of 3D7/IC was reported from Non-CHT in351-375 bp group (17.7%) and from CHT (8.5%) in 501-525 bp group respectively (Figure 7.19).

GLURP PCR and clones

All of the130 samples were positive for GLURP PCR. Thus positivity rate was 100%. A total of 141clones belonging to 13 allelic families were found by GLURP specific PCR of which 72(51.1%) were from CHT areas and remaining 69 (48.9%) were from non-CHT areas. Thus, number of MSP2 clone per positive samples was 1.04in CHT areas and 1.13 in non-CHT areas. Allelic families were ranged in between 400-1050 bp with highest frequency (26.9%) in 551-600 bp groups in overall data, non-CHT areas 16.2% and10.8% in CHT along with 901-950 bp group respectively (Table 7.12 and Figure 7.21-7.22).

Multiplicity of Infection (MOI)

Mean multiplicity of infection (MOI) and genetic diversity of MSP1, MSP2 and GLURP were calculated following the formula provided in the Material and Methods section. MOI of MSP1, MSP2 and GLURP was 1.51, 1.25 and1.08 respectively in overall samples 1.44, 1.29 and1.11 respectively in non-CHT areas and 1.58, 1.22 and 1.04 respectively in CHT areas (Table 7.13).

The expected heterozygosity (H_E)

The expected heterozygosity (H_E) of MSP1, MSP2 and GLURP were calculated following the formula provided in the Material and Methods section. H_E of MSP1, MSP2 and GLURP in non-CHT areas was found 0.84, 0.90 and0.78 respectively. These values for CHT areas was 0.76, 0.93 and 0.86 respectively and in over all data 0.80, 0.93 and 0.83 respectively (Table 7.13).

CHAPTER 10

CONCLUSION AND RECOMMENDATION

CONCLUSION

In conclusion it can be stated that a decreasing trend has been seen for malaria case burden in Bangladesh during 2010-13 compare to previous years. However, Chittagong Hill Tracts still representing majority of the malaria cases (>80%) of the country. Most pathogenic malaria caused by *Plasmodium falciparum* parasite remains highly prevalent in all the endemic districts except for Kurigram. A district seasonality of malaria incidence was observed during the study period. Malaria got high peak in between May-October although cases can be found throughout the year.

Due to ongoing malaria control activities people in endemic areas have better knowledge on malaria than non endemic areas. However, the role of the female *Anopheles* mosquitoes in transmitting malaria parasites was poorly known. There is a relationship between years of schooling with higher knowledge aboutn malaria. People rely mostly on bed nets as a preventive measure against mosquito bite. Excellent bed net coverage was found in the household level and people trust on bed nets for the protection from malaria vectors. People usually prefer to get medical attention for malaria infection from village doctors or private health providers. People will also prefer to take allopathic treatment in case they get infected by malaria.

In the present experiment the newly developed real-time PCR was found highly sensitive for detecting even a single malaria parasite from the clinical samples. Furthermore, use of SYBR Green dye instead of Probe/TaqMan in the real-time PCR platform would reduce its operational cost. The newly developed real-time PCR assay had high sensitivity and specificity for the detection of *P. falciparum* in clinical isolates in comparison with microscopy. This result is also comparable to other similar studies in global context.

A new primer set was designed for the LAMP method developed in the present study. The LAMP primer sets assessed in the present study were able to detect clinical *falciparum* malaria with great accuracy in comparison with microscopy and real-time PCR. LAMP with newly designed primers was found more sensitive than a previously described LAMP method. Newly developed LAMP method was found highly

sensitive and specific as compared with microscopy and real-time PCR. Furthermore, addition of HNB also improved its detection process as evident in the present study.

In the present study, it was found that high level of genetic diversity existed among MSP1, MSP2 and GLURP allelic families of *P. falciparum* in Bangladesh. Diversity of these three markers in Bangladesh was found higher than reported earlier in Southeast Asia/Pacific region but quite comparable to some African countries. There was no significant difference in genetic diversity MSP1, MSP2 and GLURP allelic families of *P. falciparum* among endemic areas (CHT and non-CHT) of Bangladesh.

RECOMMENDATION

Following recommendation can be made from the present study:

- There should be a strong commitment by all stakeholders of both GOB and NGOs to control malaria in Bangladesh.
- More effort is required to control malaria specially in Chittagong hill tracts districts.
- Instead of relaying a single vector control method (ITN/LLIN) an integrated vector control program should be adopted in urgent basis.
- Although local people know about malaria and the use of bed net. But there is a big gap between the actual knowledge and practices for mosquito control, proper treatment and prevention of malaria.
- Increasing of awareness campaign and advocacy for malaria control in endemic areas and installation of such champing in non-endemic areas through mass media are highly required.
- Malaria control program needs to address these gaps while designing health education interventions and specially should target the poor and the semiliterate, as these groups had lower level of knowledge compared to their counterparts.
- Health education interventions should be designed to upgrade the existing knowledge and awareness level of vulnerable population as well as their current treatment-seeking practices and should be implemented the programme for sufficient length of time to be effective.
- SYBR Green-based Real-Time PCR could be a useful tool for a reference setting to detect malaria infection in subclinical/asymptomatic cases.

- LAMP method has a potential to become a point-of-care molecular method in the field level and can be used to detect malaria infection in subclinical/asymptomatic cases in local scale.

- There is a need to monitoring closely of the clinical efficacy of existing anti-malarial drugs used in Bangladesh.

- Molecular markers (*MSP1*, *MSP2* and *GLURP*) used in the present study can be used to monitor efficacy of current anti-malaria drugs in Bangladesh as well as to test efficacy of newly developed anti-malaria drug or vaccine in Bangladesh.

CHAPTER 11

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APPENDIX

Questionnaire for KAP

Epidemiology of malaria in Bangladesh: detection and identification of genetic and molecular variation in *Plasmodium falciparum*

Demographic Characteristics of the respondents :

No	Question	Answer	Remarks
1.	Name of the respondent		
2.	Age		
3.	Sex	01=M 02= F	
4.	Marital Status	01=Unmarried 02=Married 03=Widower/Widow 04= Separated 05= Divorced 88= NA	
5.	Education	00=No formal ed. 01= Primary 02=SSC 03=HSC 04=Graduate 05=Master 88= NA	
6.	Number of Family Member		
7.	Occupation	00=Unemployed 01=Farmer 02=Daily labor 03=Service 04=Business 05= Technician 06=House work 07= Student 88= NA	
8.	Religion	01=Islam 02=Hindu 03=Buddhist 04=Christ	
9.	Tribe	01=Bengali 02=Tribal	

Status of the Living Room:

Main (largest, well) living room	Roof	Partition	Floor	Comment
	1= Straw/ Thatch 2=Tin 3=Concrete/cement 99= Others (specify) ……	1= Jut stick/bamboo 2= Tin 3= Concrete/Cement 4= Mud 99= Others (specify) ……	1= Mud 2= Cemented 3= Semi-cemented 99= Others (specify) …….	1= Jhupri 2= Not Jhupri

Perceived HH Economic Status:

	Question	Answer
1.	What is monthly income of the family?	……. Tk
2.	What was the household's economic status you feel after observing last one year's income and expenditure from different sources?	All the year deficient……1 Deficient sometimes …….2 Not deficient nor surplus ……..3 Surplus ……..4
3.	(What kind of economic changes occurred during last one year?	Condition improved ……..1 Not improved…….2 Condition deteriorated ……..3

Practices / Awareness:

	Question	Answer	Remarks
1.	When did you last enter the house previous night?		
2.	When did you go to bed last night?		
3.	Where did you spend time last afternoon?	1=Inside; 2=Outside; 3=Market; 4=Tea Stall	
4.	Where do you spend most of the day time?	1=inside, 2=outside	
5.	Where do you spend most of the night time?	1=inside, 2=outside	

Malaria awareness:

	Question	Answer	Remarks
1.	Do you or any member of your family have malaria?	1=Yes; 2=No	
2.	If yes, how many days ago?	1=less than one month, 2=less than 3 months, 3=more than 6 months, 4=more than one year	
3.	Have you heard about malaria?	1= Yes; 2= No	
4.	Why someone gets malaria?	1=Mosquito bites; 2=Fly/insect bites; 3=Lack of cleanliness; 4=others (specify)	
5.	How do you know that you have got malaria? (Symptoms of malaria)	1=Fever with rigor; 2= intermittent fever; 3= Fever with sweating; 4=others (specify)	
6.	How malaria is transmitted from one to another?	1= Bitten by any mosquito; 2= Bitten by a mosquito which fed by a malaria patient; 3=others (specify)	
7.	How malaria could be prevented?	1=Limiting mosquito's breeding places; 2= Bed net; 3= Mosquito coil/ointment; 4= ITN; 5= others (specify)	
8.	How would you treat if you get malaria?	1= Allopathic; 2=Kabiraji/ unani; 3=Spiritual; 4=Homeopathic; 5= others (specify)	
9.	Where you can get this treatment?	1= Govt Hospital; 2= Private health center; 3=Village doctor; 4=Drug seller; 5= others (specify)	
10.	How did you obtain this information?	1= Govt health worker; 2=Non-Govt health worker; 3=Radio; 4=Newspaper; 5=Television; 6=Poster, leaflets etc ; 7= others (specify)	
11.	Who are providing malaria treatment in your area?		

Information about trapping room:

1.	How many people slept in the room at the trapping night?	
2.	How many people slept under bed net?	
3.	How many people slept without bed net?	

Information on bet net and use of bed net:

1	How many bed nets are there in your house?	No: _____ ; 0= No
2.	In which season you use bednets	1=Summer/2=Winter/3=All the year
3.	Do all of your family members sleep under bed net?	1= Yes; 2= No; 7=Seldom/irregularly
4.	How many of your family members sleep under bed net regularly?	_____ Persons
5.	How many of your family member sleep under bed net last night?	_____ Persons
6.	Age distribution of the family member who slept under bed net last night. < Less than 1 year 1-4 years 5-14 years More than 15 years	 _____ Persons _____ Persons _____ Persons _____ Persons
7.	Has your bed net been treated with medicine within last 6 months?	_____ Persons Not applicable=0
8.	How many bed nets were treated with medicine within last 6 months?	1= Yes; 2= No; 9=Don't know

Use of Insecticide:

	Question	**Answer**	**Remarks**
1.	Do you use anything for mosquito control at night?	1=Yes/2=No	
2.	If yes, what kind of?	1=Mosquito coil/2=Dhup/3=Smoke/4=Others	
3.	If yes, how often you use?	1=Always/2=sometime/3=only in summer months	
4.	What is the monthly cost of the insecticide?	1=below 100, 2= below 200, 3=more than 200	

About Domestic Animal:

	Question	**Answer**	**Remarks**
1.	Do you have domestic animals?	Yes/No	
2.	If yes, How many? a. Cattle? b. Goat? c. Chicken? d. Duck? e. Dog? f. Cat	 a. b. c. d. e. f.	