MALARIA EPIDEMIOLOGY IN DISTRICT BANNU, KHYBER PAKHTUNKHWA: IDENTIFYING GENOTYPES OF *PLASMODIUM VIVAX* IN THE REGION

PH.D THESIS

BY

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UNIVERSITY OF PESHAWAR
Session (2009-2010)
MALARIA EPIDEMIOLOGY IN DISTRICT BANNU, KHYBER PAKHTUNKHWA: IDENTIFYING GENOTYPES OF PLASMODIUM VIVAX IN THE REGION

THESIS SUBMITTED TO THE DEPARTMENT OF ZOOLOGY, UNIVERSITY OF PESHAWAR, PESHAWAR, PAKISTAN IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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AUTHOR’S DECLARATION

I, Fatima Jahan hereby state that my Ph.D thesis titled “Malaria Epidemiology in District Bannu, Khyber Pakhtunkhwa: Identifying Genotypes of Plasmodium Vivax in the Region” is my own work and has not been submitted to previously by me for taking any degree from this university of Peshawar, Pakistan or anywhere else in the country / world.

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Student Name

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ABSTRACT

Malaria is the fourth leading cause of deaths among communicable diseases in Pakistan. Federally Administered Tribal Areas (FATA), Baluchistan and Khyber Pakhtunkhwa provinces have one of the highest Annual parasite incidence (API) within the country. Present study aimed at elucidating epidemiology and comparing performance of several diagnostic procedures in Bannu, a highly endemic district (API of 1.6-3.5 per 1000) of Khyber Pakhtunkhwa.

The aim of this PhD project was to generate current information on parasite prevalence through active antigen detection and also investigated the risk factors of malaria. In addition, genetic diversity among existing *P. vivax* strain was also investigated. A total of 2033 blood samples of suspected cases were collected and processed through three diagnostic technique including microscopy, RDT and parasite species specific PCR. A questionnaire was administered to collect household and individual based information to determine the potential risk factors of malaria.

The present study was performed from 2012 to 2013. Samples were collected from suspected individuals of rural and urban areas visiting laboratory. Microscopy and Rapid Diagnostic Test was used on the spot for detection of *Plasmodium* species. A total of 2033 individuals were recruited, of whom 21.1% (N=429) were positive for malaria by at least one method. Overall, positivity detected by PCR was 30.5% (95/311) followed by 17.7% by microscopy (359/2033) and 16.4% by RDT (266/1618). *Plasmodium vivax* (16.9%, N=343) was the detected as the dominant species followed by *Plasmodium falciparum* (2.3%, N=47) and mixed infections (1.2%, N=39). Microscopy and RDT (Cohen's kappa k=0.968, 15 p=<0.0001, McNemar test p=0.069)
displayed significant agreement with each other, suggesting that RDT may be a useful alternative to microscopy in the field.

Satisfactory health, sleeping inside room, presence of health care facility in vicinity (at an accessible range from home), living in upper middle class and in concrete houses significantly reduced malaria risk. On the other hand, low literacy level, presence of domestic animals indoors and a diagnosis recommended by clinician increased the disease risk.

Mitochondrial genome sequencing of *Plasmodium vivax* revealed the existence of significant number of SNPs in coding and non-coding region of the genome while population structure analysis shown the presence of strain which are closely related to the strains of other regions. Population genetic study identify the significant mutation in the genome. It also identifies the large evolutionary distance between Pakistani and other regions *P. vivax* strains.

This data will help to provide beneficial and up-to-date information to manage control activities in the study area. Appropriate management of identified risk factors can benefit in reducing the prevalence of malaria in Bannu and its peripheries.
### ABBREVIATIONS

<table>
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<tr>
<td>KPK</td>
<td>Khyber Pakhtunkhwa</td>
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<td>WHO</td>
<td>World Health Organization</td>
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<td>RH</td>
<td>Relative humidity</td>
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<tr>
<td>Pak</td>
<td>Pakistan</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>TBE</td>
<td>Tris-Borate EDTA</td>
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<tr>
<td>HWE</td>
<td>Hardy Weinberg equilibrium</td>
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<tr>
<td>RBCs</td>
<td>Red blood cells</td>
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<td>WCH</td>
<td>Women and children hospital</td>
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<td>CDC</td>
<td>Center for Disease and Control</td>
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Chapter 1  INTRODUCTION

1.1 Malaria

Malaria is a parasitic disease caused by protozoan of the genus *Plasmodium* of the phylum Apicomplexa which was described in 1885 by Ettore Marchiafava and Angelo Celli. The five known human malaria species *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi* which were identified to infect humans (Omar et al., 2001a, Omar et al., 2001b) while *Plasmodium vivax* is the main cause of malaria morbidity outside Africa (Carlton et al., 2008) and has historically been mostly neglected in control, relatively because of its lower virulence than *P. falciparum* (Price et al., 2007).

The most serious and sometimes fatal type of malaria is caused by *Plasmodium falciparum*. Approximately 80 – 90% of *P. vivax* burden is concentrated in the Middle East, Asia and Central & South America (Guerra et al., 2006). *Plasmodium falciparum* is responsible for severe form of infection and cause approximately 90% of deaths and 80% of human infections, with symptoms arising after approximately 6 to 14 days, including fever, vomiting, arthralgia, shivering, convulsions, hemoglobinuria and anemia caused by hemolysis (Beare et al., 2006).

Other clinical features include splenomegaly, anemia, thrombocytopenia, hypoglycemia and pulmonary or renal dysfunction. In severe infections neurological damage coma and even death can be caused by rosetting of the red blood cells, leading to impaired micro-vascular blood flow in the brain, and the release of cytokines such as TNF which can in turn trigger the release of harmful substances such as nitric oxide which affect the central nervous system (CNS) causing neurologic deficits, cognitive sequelae, damage of brain (Mayxay et al., 2004) and epilepsy (neurocognitive sequelae are defined as impairment of neurologic or cognitive function). The neurologic impairment caused by *P. falciparum* consists of loss of function in motor, including
coordination, speech, vision, and hearing domains, as well as epilepsy) (Omar et al., 2001a, Omar et al., 2001b).

1.2 History of malaria

During second century, ancient Nei Ching (the canon of Medicine) (about 4700 BCE) the initial malaria-like symptoms were recorded. These writing also consist of various malaria treatment, one of them is the use of Qinghao (Artemisia annua) plant for fever reduction (Hsu, 2006). Artemisinin, the active ingredient of the Qinghao plant, was not isolated until 1971 by Chinese scientists. Artemisinin drug products are used today as antimalarial drugs in areas where there is resistance to chloroquine (MalariaSite., 2017, CDC, 2017b).

In the early 17th century the Spanish Jesuit missionaries recognized the use of another medication found in the bark of tree in Peru. The tree was named Cinchona after usage of its bark which cured the fever of countess of Cinchon, the wife of viceroy of Peru. The bark was called Peruvian bark. The recently used antimalarial quinine is the derived from Peruvian bark (CDC, 2017b).

In the 4th century BCE Hippocrates documented (in the sanskrit medical treatise “Susruta”) the signs of malaria along with time of year and place where the victim fell ill, he was the first to differentiate the recurrent fever of malaria from continual fever of other infectious diseases. For a long time, malaria was associated with swamps and it was a concept that it is transmitted by wind, modern English term malaria is derived from Italian malaria from malaria, literally “bad air”. An Italian Physician Francisco Torti (1658-1741), used the term malaria for the first time. In 1880, a French Army Physician Charles Louis Alphonse Laveran discovered the causative agent of human malaria in the patient blood, received Nobel Prize for medicine and physiology in 1907. Later, Laveran investigated the presence of malaria parasite outside the human body.
He deduced that “the marsh fever parasite must undergo one phase of its development in mosquitoes and be inoculated into humans by their bites” (Bloland, 2001).

In 1886 Camillo Golgi concluded from his study that there are at least two forms of malaria. The one called tertian which produced fever on alternative day. Another type was quartan, produced fever every third day. In 1890 the two Italian investigators, Giovanni Batista Grassi and Raimondo Filetti introduced the terms *Plasmodium vivax* and *Plasmodium malariae*. The third malaria parasite *P. falciparum* was named by American William H. Welch in 1897 while reconsidering the Laveran’s work. Finally, in 1922, John W. W. Stephens found a fourth human malaria parasite *P. ovale*. In 1897 a British officer Ronald Ross revealed that malaria parasite is transmitted by dappled wing brown Anopheles mosquito and demonstrate that parasite can be transferred to mosquito by introducing parasite free mosquitoes into the rooms of malaria infected patients. The *Plasmodium* life cycle was first described by Giovanni Batista Grassi in 1899 (CDC, 2017b).

Almost 60 years after the discovery of erythrocytic stages of *Plasmodium*, in 1948 the tissue stage of primates and human *Plasmodium* were detected in the livers of rhesus monkeys infected with *P. cynomolgi* Sporozoites by Shortt, Garnham and colleagues at the Ross institute in London. Later the Shortt and colleagues describe the complete life history of *P. falciparum*. With progress of molecular & computer technologies and the discovery of in vitro culturing of falciparum, many studies defined the malaria parasite classification, structure, Physiology, molecular composition, biochemistry, immunology and genetics (Trager and Jensen, 2005, Haynes et al., 1976).

The chief advancement in the present and future malaria research is the whole genome sequencing of some *Plasmodium* species, first of *Plasmodium falciparum* (Gardner et al., 2002) and later *Plasmodium vivax, Plasmodium knowlesi, Plasmodium berghei,*
Plasmodium chabaudi & Plasmodium yoelii (Aurrecoechea et al., 2009). The molecular innovation led to a rapid extension of research into the structure and function of proteins expressed during life cycle, use of novel techniques for studying genetic mutation and provides a plenty of data for new drug and vaccine targets.

1.3 Plasmodium; the parasite

Plasmodium shares some of the features with eukaryotes while some of its characteristics are unique to phylum or genus. Like eukaryotes it contain a nucleus with a single copy of genome in almost all parts of life cycle except in the mid gut of the insect host where doubling of genome occurs for a brief sexual exchange (Obado et al., 2016). Endoplasmic reticulum are attached with nucleus which functions like in other eukaryotes. Proteins are transferred from the endoplasmic reticulum to the Golgi apparatus which generally consists of a single membrane-bound compartment in Apicomplexans (Jimenez-Ruiz et al., 2016). From here proteins are transported to various cellular compartments or to the cell surface (Jimenez-Ruiz et al., 2016).

In human Plasmodium, there are four developmental stages in humans (hepatic schizonts and then intraerythrocytic trophozoites, schizonts and gamonts) and three stages in Anopheles vector (ookinetes, oocysts and sporozoites). Liver schizonts are clusters of small basophilic bodies (merozoite nuclei), 40-80µm in diameter when mature, present in liver cells. The blood stages consist of small circular trophozoites (ring form) of 1-2µm diameter, unstructured multinucleate schizonts measuring up to 7-8µm in size, the micro and macro gametocytes ranges from 7-14µm in length. The morphology of blood stages is species specific. Micro-gametocytes have a larger more diffuse nucleus (ready for gamete production) while macro-gametocytes have darker-staining cytoplasm (plentiful ribosomes for protein synthesis) (Qiagen, 2013).
In mosquito, the elongated microgametes (15-25µm in length) formed by exflagellation fertilize the rounded female gametes and form a motile Ookinetes (15-20 x 2-5µm) which travels through the gut wall form rounded oocysts (up to 50µm in diameter) on the outer surface. Thousands of thin elongated Sporozoites (~15µm long) were formed which migrated to salivary glands (www.parasite.org.au) (Bloland, 2001, CDC, 2017b)

Morphologically sporozoite is about 10 µm to 15 µm long by 1 µm in diameter and have a pellicle consist of a thin outer membrane, a doubled inner membrane, and a layer of sub-pellicular microtubules. There are three polar rings and rhoptries are long, extending to the mid-portion of the organism while rest of the cytoplasm is occupied by Micronemes. Apparently a nonfunctional cytostome is present and there is a mitochondrion at the posterior end of the sporozoite (Aikawa & Sterling, 1974).

The unusual characteristics of Plasmodium in comparison to general eukaryotes is the presence of specific apical secretory organelles (Rhoptries, Micronemes and dense granules) that secrete special protein required for infecting new host and polar rings near the apical end. The secretion by apical secretory organelles are unique to Apicomplexans (Counihan et al., 2013).

1.3.1  Plasmodium Genome

Plasmodium have 14 chromosomes in the nucleus along with genetic material in the mitochondrion and in the apicoplast. The chromosomes vary from 500 kb to 3.5 mb in length. The apicoplast is involved in isoprenoid metabolism, Fe-S cluster synthesis, fatty acid synthesis, and phospholipid biosynthesis. On a molecular level, the parasite damages red blood cells using plasmepsin enzymes aspartic acid proteases which degrade hemoglobin Evolution of Plasmodium (van Dooren and Striepen, 2013).
1.3.2 Evolution of *Plasmodium*

The ancestors of *Plasmodium* probably existed at least half a billion years ago. The molecular studies provide strong evidence that the pre-parasitic ancestor of *Plasmodium* was chloroplast containing free living protozoan which adapted for the gut habitat of aquatic invertebrates (insect larvae, including those of early Dipterans, the taxonomic order to which mosquitoes and other blood-sucking flies belong) and later that single celled organism probably had obligate sexual reproduction in the lumen of mid-gut of host. This pre-malaria parasite acquired an asexual and intracellular reproduction called schizogony (schizogony in the RBCs of humans causes the clinical manifestations of malaria) and increase its division potential. During that period the parasite achieved two host life cycle, an adaptation to the blood feeding habits of insect hosts (Carter et al., 2000).

The complexities of the evolutionary relationships, geographic distribution, prevalence rates, and mammalian host and vector associations of *Plasmodium* parasites were recently illuminated though it was discovered almost 100 years ago. Though evolutionary origin of human *Plasmodium falciparum* and *P. vivax* has now been elucidated but nothing is known about the mechanism that led to their emergence, such info is very essential for understanding how ape parasite crossed the species barrier and whether this process can occur again. The great barrier for efficient analysis of ape malaria parasite is the lack of in vitro culture system for *Plasmodium* parasite, but the whole genome sequencing of parasite from infected blood is the valuable step towards exploration of biology of ape *Plasmodium* (Otto et al., 2014, Sundararaman et al., 2016). This analysis revealed several surprising findings such as horizontal transfer of invasion genes among ape Laverania parasite species (Sundararaman et al., 2016).
In past *Plasmodium* diverged into two groups, one infected mammals and other having bird or reptile hosts. Within mammalian division *Plasmodium falciparum* and *P. reichenowi* form a unique subgroup but split off early and diverged to rodents and other primate parasites. It is evident from molecular studies that divergence of *Plasmodium falciparum* and *P. reichenowi* occurred about 6-8 million years ago, when the chimpanzee and human lineages were diverged (Martinsen et al., 2008).

One of the old hypotheses suggested that humans and chimpanzees inherited the *Plasmodium falciparum*-like infection from the common ancestor and for millions of years these parasites co-evolved with their respective host species (Escalante and Ayala, 1994) while *Plasmodium vivax* was believed to evolve several hundred years ago after the species transmission of a parasite from a macaque in southeastern Asia (Escalante et al., 2005, Jongwutiwes et al., 2005, Mu et al., 2005, Neafsey et al., 2012). Both theories have recently been contradicted after the characterization of high number of additional malaria parasites from the African apes. Unambiguously it is now proved that *Plasmodium falciparum* infection is relatively new for human population as it arose after acquiring parasite from a gorilla, probably within past 10,000 years (Liu et al., 2010, Sundararaman et al., 2016). Likewise, *Plasmodium vivax* didn’t emerge in Asia but presents a bottleneck lineage that absconded out of Africa afore the spread of Duffy negativity which reduced African humans resistant to *P. vivax* (Liu et al., 2014).

Based on presence of homozygous hemoglobin C and RBC Duffy negativity alleles that confer protection against *P. falciparum* and *P. vivax* respectively, it is believed that most of the present-day *Plasmodium* population may have their origin in West Africa (*P. falciparum*) and West and Central Africa (*P. vivax*) (Makanga et al., 2016).

As *falciparum* was found to be very closely related to *Plasmodium reichenowi* of chimpanzees and both are more closely related to bird’s malaria parasite as compared
to other mammals. The ancestry of these parasites probably occurred around 130 million years ago, almost the same period of origin of the two-host life cycle involving blood feeding insects and land vertebrates. The separation of these lines probably occurred only 4-10 million year ago, coinciding the era of human divergence from African great apes. The modern phylogenetic studies of *Plasmodium falciparum* reveal that all existing *falciparum* populations were originated from *P. reichenowi* probably by a song host transfer, 2-3 million years ago (Rich et al., 2009, Carter and Mendis, 2002) while the present lethal strain of *Plasmodium falciparum* may have emerged within last 5-10 thousand years, after the development of agriculture in Africa (Carter and Mendis, 2002).

Many of the molecular studies disproved the *Plasmodium falciparum* origin from chimpanzee, bonobo or ancient human origin. The mitochondrial, apicoplast and nuclear genome proved the monophyletic lineage of human *P falciparum* within the gorilla parasite radiation (Krief et al., 2010, Liu et al., 2010, Prugnolle et al., 2010).

Another human *Plasmodium* species (*malariae, ovale* and *vivax*) diverged 100 million years ago along the mammalian *Plasmodium. P. ovale* is the only known surviving representative of its lineage and caused malaria in humans only. The ancestors of both human and African great apes were parasitized by *P. malariae* and it had the ability to parasitize and cross-infect both host lines as they diverged around five million years ago. *P. malariae* (naturally infect the West Africa chimpanzees) and *P. brazilianum* (infect new world monkeys in central and south America) were morphologically indistinguishable. Like *P. ovale, P. malariae* is the only surviving member of its line. *P. vivax*, closely related to *P. shwetzi* (parasite of African great apes), belongs to a group of malaria parasites like *P. cynomolgi*, that infect monkeys (Daneshvar et al., 2009).
The divergence time of *P. vivax* from *P. cynomolgi* is about 2-3 million years ago. Several cases of *Plasmodium knowlesi* infection (zoonotic from macaque monkeys) have been recently reported from Southeast Asia, including Thailand, Malaysia, Vietnam, Phillipines and Myanmar (Daneshvar et al., 2009, Singh et al., 2004, Cox-Singh et al., 2008, Peters and Van Noorden, 2009, Rich et al., 2009, Putapornpit et al., 2011).

The genome sequencing of additional species such as ape *P. vivax* and *P. praefalciparum* will provide patterns for systematic studies and in vitro genome manipulation to compare the function of key proteins among the various ape and human *Plasmodium* spp. Population genomic studies of ape Laverania parasites may also inform ongoing malaria vaccine development efforts by identifying antigens that are under strong immune selection pressure in apes as well as humans (Ochola et al., 2010, Amambua-Ngwa et al., 2012). The characterization of transmitting vector of Ape malaria parasites and to find the degree of human exposure to these parasites through the bite of infected mosquitoes, will be of utmost importance. A careful study of antibody responses to pre-erythrocytic stages could provide answer to this question. Finally, *P. ovale* and *P. malariae* like sequences have been detected in African great apes (Duval et al., 2010, Krief et al., 2010, Liu et al., 2010), and additional work is required to ascertain the relationship of these parasites to their human counterparts.

For the past 80 years, 4 *Plasmodium* species (*P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*) are known to cause human malaria with *P. falciparum* being responsible for more severe outcomes (Kantele and Jokiranta, 2011). The molecular analysis of small subunit rRNA gene of these species suggested that they are the members of separate phylogenetic lineages of other animal’s malaria parasites. According to this analysis *Plasmodium falciparum* is closely related to bird parasites (*P. gallinaceum* and *P.*
lophurae), *P. malariae* have its own lineage while *P. vivax* showed close association to several other primates' species due to insufficient data the placement of *P. ovale* in respective lineage is difficult (Waters et al., 1993).

Other study of mitochondrial cytochrome b gene placed the *P. falciparum* in a clade with *P. reichenowi* (from chimpanzees) not related to other *Plasmodium* of mammals. This study also support placement of *Hepatocystis spp.* And *Haemoproteus* in separate clades with different species of *Plasmodium*, thus making genus *Plasmodium* paraphyletic (Perkins and Schall, 2002, Perlmann et al., 2000). Taxonomy in parasitology before DNA based methods was always problematic, and revisions are continuing, leaving many obsolete names for *Plasmodium* species that infect humans (Coatney and Roudabush, 1936). Recently, a fifth *Plasmodium* species *P. knowlesi* has been documented as a human malaria parasite which was formerly known to cause malaria only in macaques (Kantele and Jokiranta, 2011).
Figure 1.1 Evolutionary relationships of *Plasmodium* spp.

Colours highlight *Plasmodium* spp. that infect humans (red), chimpanzees (blue) and gorillas (green). Four groups of *Plasmodium* spp. are shown, with subgenus designations indicated for primate parasites. The phylogeny was estimated by maximum likelihood analysis of 2.4 kb of the mitochondrial genome; the scale bar indicates 0.03 substitutions per site.

### 1.3.3 Taxonomy

*Plasmodium* belongs family Plasmodiidae (Levine, 1988), order Haemospridia and phylum Apicomplexa. There are 450 known species in the order (www.wikipedia.com). The genus *Plasmodium* was generated in 1885 by Marchiafava and Celli (Chavatte et al., 2007). The family Plasmodiidae comprises genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon*, which are malaria and malaria like organisms. When in host cells *Plasmodium*, *Haemoproteus* commonly produce a Hemozoin pigment from host hemoglobin, differentiate them from the closely related *Leucocytozoon*, which does not produce Hemozoin (Robert and Janovy, 2009).
Genus *Plasmodium* was divided by Garnham (Garnham, 1966) into nine sub genera, of which 3 occurs in mammals, 4 in birds and 2 in lizards. Most *Plasmodium* species are parasite of birds and others are parasite of rodents, primates and reptiles (Robert and Janovy, 2009).

1.3.4 **Reservoirs of Plasmodium:**

In tropical Africa, Chimpanzees may carry the *P. malariae*. No other animal reservoir of human Plasmodia is known to exist. Asymptomatic person can be a reservoir who harbors the sufficient number of viable, mature, male and female gametocytes in blood (Park, 1998).

1.4 **Vector of Malaria**

There are about 3500 species of mosquitoes which are categorized into 41 genera. The females of genus *Anopheles* are the vector of human malaria. Among 430 species of *Anopheles* only 30-40 are involved in malaria transmission. Malaria transmitting Anopheline mosquitoes are found both in endemic and non-endemic countries thus the non-endemic countries are always at risk of re-introduction of malaria. Most of the *Anopheles* are crepuscular (active at dusk or dawn) or nocturnal (active at night). They may be endophagic (feed indoor) or exophagic (feed outdoor) in feeding habit (CDC, 2017b).

Depending on resting behavior mosquitoes may be exophilic (rest outdoor) or endophilic (rest indoor). The nocturnal and endophagic mosquitoes can be controlled by use of insecticide treated nets (ITNs) or by improving the house construction to prevent the mosquito entry in the houses. Endophilic *Anopheles* can be reduced by indoor spray of residual insecticides. Breeding sites destruction is the effective way for reducing the exophagic/exophilic mosquitoes. The survival of vector, ambient temperature and humidity play an important role in the developmental of parasite inside
the *Anopheles*. In contrast to humans the parasite doesn’t cause any harm to mosquitoes (CDC, 2017b, WHO, 2017).

*Anopheles* feed at night although few also feed at daytime. *Anopheline* mosquitoes rest with the body sloping forwards. Their breeding sites vary and include permanent or temporary pools, swamps, seepages, rice fields, tree holes, ditches and reservoirs with some requiring sunlight and others shade for breeding (Cheesbrough, 1987).

1.4.1 **Distribution of malaria vector**

Figure 1.2 Map of the world showing the distribution of predominant malaria vector (CDC, 2017a).

1.5 Transmission & pathogenesis

1.5.1 Vector and hosts

Principal mode of spread of malaria is by the bites of female *Anopheles* mosquito. Of more than 500 species of *Anopheles*, only 80 were reported for malaria transmission, of which about 40 are significant vectors. The female *Anopheline* mosquito needs a blood meal and habitat for laying eggs. Some vectors have anthropophilic behaviours, which include preferences for humans as a source of blood meal and endophily (the tendency to enter and rest inside houses) (Besansky et al., 2004, Bannister, 2009). Every continent is reported with its own species of these mosquitoes: *An. Gambiae*, *A. arabiensis*, *A. funestus* are found in tropical Africa (Besansky et al., 2004, Coetzee et al., 2000, Lindsay and Martens, 1998), *An. freeborni* in North America, *An. culicifacies*, *An. fluviatilis*, *An. minimus*, *An. philippinensis*, *An. stephensi*, and *An. sundaicus* in the
Indian subcontinent (CDC, 2017b). *An. leucosphyrus, An. latens, An. cracens, An. hackeri, An. dirus* etc., have been identified as the vectors for the transmission of *P. knowlesi* (Van den Eede et al., 2010).

Most of the Anopheline mosquitoes have been characterized as anthropophilic (prefer human blood meal), endophagic (bite indoors), and nocturnal (bite at night) with peak biting at midnight, between 11 pm and 2 am while some shows the endophily (tendency to enter houses and rest inside) (Besansky et al., 2004, CDC, 2017b). The blood meal from a vertebrate host is essential for the female mosquitoes to nourish their eggs.

The mosquitoes find their host by seeking visual, thermal, and olfactory stimuli, the most important mosquito attractants are carbon dioxide, lactic acid, skin temperature, and moisture. As these stimuli varies from person to person, the exposure of individuals to mosquito varies according to strength of these stimuli (Fradin, 1998, Murray et al., 2003). Females Anopheles transmit the infection through biting the healthy individual (Robert and Janovy, 2009).

Like all other mosquitoes, each *Anopheles species* breed in water and each have specific breeding site, feeding pattern and resting place. Ambient temperature is mandatory for completion of life cycle of both vector and parasite (Craig and Sharp, 1997). As the survival of the mosquito is rainfall and temperature dependent (Reiter, 2001, Zhou et al., 2004, Alsop, 2007), therefore any change in the range would affect the mosquito bionomics. A study conducted on temporal correlation between malaria and meteorological factors in Tibet, it was found that the relative humidity (RH) was the greatest influencing factor, which affected the mosquito survival directly (Huang et al., 2011).
1.5.2 Transmission of malaria:

Principal mode of malaria transmission is the bite of Anopheles mosquito. In other modes of transmission includes the direct inoculation of asexual forms into the blood and the pre-erythrocytic development of organism does not occurs in the liver. Other way of transmission is the transfer of parasite from infected mother to the child trans-placentally or either during labor called congenital malaria. According to recent studies the congenital malaria ranges between 8%-33% from both endemic and non-endemic regions. Transfusion is another way for transmission of malaria. The administration of antimalarial drugs to transfusion recipients may help to prevent transmission. Cases of malaria transmission through needle-stick injuries, accidentally among health care professionals (some even fatal) or due to needle sharing among drug addicts, have also been reported (Bartoloni and Zammarchi, 2012)

1.5.3 Life cycle:

The life cycle of *Plasmodium* alternates between vertebrate and invertebrate hosts with sexual phase in the invertebrate host (mosquito).

1.5.3.1 Invertebrate phase (definitive host)

Along with blood meal Gametocytes are ingested by Female mosquito (Anopheles) and reached up to mid gut, where further development starts. In the mid gut the micro and macro gametocytes escape from the RBCs and develops into male and female gametes. Male gametocytes multiply rapidly into many motile flagellated microgametocytes and each can fertilize a female gamete to form a zygote (the only diploid stage of *Plasmodium* life cycle). After 18 hours of blood meal the zygotes develop in Ookinetes (fertilized motile cell). The ookinete penetrates the epithelial layer of gut wall and encysted in the gut wall as rounded oocyst. Within oocyst sporogony occurs in which haploid
sporozoites are produced through meiosis (Sinden and Hartley, 1985, Torii et al., 1992). After maturation, the oocyst rupture and Sporozoites migrate to salivary glands where it completes remaining developmental process (Rosenberg and Rungsiwongse, 1991). The average duration of sporogony is round about two weeks and ranging between 1-5 weeks (depends on species and temperature). Further continual of life cycle depends on inoculation of sporozoites into human body (Bannister, 2009, Morell, 1997, Ngasala et al., 2008).

1.5.3.2 Vertebrate Phase:

1.5.3.2.1 Inoculation of sporozoites

When infected mosquito bites the human it injects about 10-100 sporozoites per blood meal. Sporozoites moves to liver and invade hepatocytes (liver cells) (CDC, 2017b).

1.5.3.2.2 Tissue Phase (Hepatic stage and Pre-erythrocytic stage)

Sporozoites enter the bloodstream or lymph and circulate to parasitize the liver or spleen of mammals and endothelial cells and macrophages (birds and reptiles). There they proliferate to form hundreds of intrusive merozoites (exo-erythrocytic phase or pre-erythrocytic). These merozoites released into the bloodstream and attack the Red blood cells (Bannister, 2009).

Inside the liver cells the sporozoites converts to trophozoites which grows and divide through Schizogony and produce thousands of merozoites (about 30,000 for Plasmodium falciparum and 10,000 for Plasmodium vivax). Duration of hepatic schizogony vary with species (about 5 day for P. falciparum, 16 days for P. malariae). In Plasmodium vivax and P. ovale the sporozoites can remain dormant in liver cells as hypnozoites, which can reactivate at variable interval and cause disease relapse. The liver schizonts rupture on maturation and release merozoites into blood stream and each occupy red blood cells (Gilles, 1933, Tuteja, 2007).
1.5.3.2.3 Erythrocytic stage

The attachment of parasite to erythrocyte is mediated by specific erythrocytes surface receptor. Inside red blood cells, the merozoites converts to trophozoites and start feeding host cell contents also form food vacuoles where they digest the host cell hemoglobin. After certain period of growth, the mature trophozoites multiply several times to form more trophozoites and invade non-infected erythrocyte. This cycle of division repeats several times to increase the number of parasite greatly. The erythrocytic cycle duration varies for each species (48 hours for *P. vivax*, *P. falciparum* and *P. ovale*, 72 hours for *P. malariae* and 24 hours for *P. knowlesi*) (Nafo Traore, 2005).

At this phase when schizonts rupture releasing merozoites and parasite by-products which cause the clinical symptoms of malaria while the merozoites invades the uninfected RBCs and erythrocytic schizogony repeats again. Some of the merozoites develops into either female gametocyte (macrogametocyte) or male gametocyte (microgametocyte) (Bannister, 2009, Ngasala, 2010, Tuteja, 2007) Continuation of life cycle now depends on fate of gametocytes being taken into the gut of female mosquito where both gametocyte (micro and macro) escape from their host cells. One of the characteristic feature of *Plasmodium falciparum* is the sequestration (adherence of infected red blood cells to endothelial cells of various organs), protects the organism from clearance system occurs in spleen (Miller et al., 1994, Treutiger et al., 1992).
Figure 1.3 Life cycle of *Plasmodium* parasite (Bannister, 2009)

The life cycle of malaria parasites. Parasites enter the vertebrate host through a mosquito bite. Sporozoites enter the skin and travel through the bloodstream to the liver, where they multiply into merozoites, which return to the blood. Merozoites infect RBCs, where they develop through several stages to produce either more merozoites, or gametocytes. Gametocytes are ingested by mosquito and become infected, continuing the life cycle.

1.5.4 Pathogenesis in vector host:

As to whether the *Plasmodium* parasites cause harm to their mosquito hosts is a matter of debate, with several contrasting opinions stressed. It does seem that mosquitoes that are carrying the malaria parasite do experience decreased life expectancy and higher mortality rates than their non-infected counterparts (Kettle, 1995). *Plasmodium* spp. cause pathogenesis to their vertebrate hosts and apparently effect the fitness of their vectors. Infected mosquitoes survive starvation comparatively better than non-infected
mosquitoes, associated with storage of high energy resources that is accumulated by mosquito during oocyst development of *Plasmodium* (Zhao et al., 2012).

Microarray analysis reported that the metabolism of infected mosquitoes is altered during rapid growth oocysts, due to suppression of several enzymes involved in carbohydrate catabolism. Additionally, enhanced expression of several insulin-like peptides was noted in infected (*Plasmodium*) mosquitoes. Impaired *Plasmodium* development due to blocking of insulin-like signaling pathway was also observed. Shortly the *Plasmodium* infection disturb the metabolic pathways in mosquitoes, thus thereby conferring a survival advantage to the insects during periods of starvation. (Zhao et al., 2012).

The viability of the mosquito vector is sometime reduced by *Plasmodium* infection specifically at the stage when Ookinetes occupied the mosquito mid gut and provoke an energy demanding immune response by the vector (Ferguson and Read, 2002, Maier et al., 1987, Michel and Kafatos, 2005). In general, *Plasmodium* is thought to reduce the overall fitness of its arthropod vector (Vernick et al., 2005).

### 1.5.5 Disease Pathogenesis in Human

Malaria is an acute febrile illness with incubation period of 7 days or longer (Vanderberg, 1980). The frequency of malarial fever varies with the length of schizogony in erythrocyte. With the first attack *P. falciparum*, irregular pattern of fever is seen unlike repeating patterns seen with tertian fever, usually no relapses occur as there are no hypnozoites are formed in *Plasmodium falciparum* (Vanderberg, 1980). The infection of RBCs by asexual forms of *Plasmodium* results in all manifestations of disease. All type of malaria display common signs such as fever and some patients may progress to the severe malaria. Though severe malaria is usually seen in *Plasmodium*
*falciparum* but severe cases and even deaths were also reported from non-*falciparum* malaria (Omer et al., 2003).

The destruction of RBCs results into most of the pathologic outcomes. Red blood cells are destroyed by release of merozoites and by spleen which first sequester the infected RBCs and then lyse them. The enlargement of spleen (the characteristic feature of malaria) is caused by congestion of sinusoids with erythrocytes along with hyperplasia of lymphocytes and macrophages (Levinson, 2014). During bursting of RBCs (containing schizonts), along with merozoites numerous substances are released in the blood such as blood cell membrane products, hemozoin pigment and toxic substances (such as GPI) which activates the macrophages and endothelial cells to secrete cytokines and inflammatory mediators (tumor necrosis factor (TNF), interferon-γ, interleukin-1, IL-6, IL-8, macrophage colony-stimulating factor, and lymphotoxin, as well as superoxide and nitric oxide). GPI tail which is common to several merozoite surface proteins such as MSP-1, MSP-2, and MSP-4, act as a key parasite toxin (Chakravorty et al., 2008, Clark and Cowden, 2003, Gowda, 2007, Mackintosh et al., 2004, Nebl et al., 2005, Pichyangkul et al., 1994).

The common manifestation of malaria such as fever, headache, rigors, vomiting and nausea, anorexia, diarrhoea, fatigue, joints and muscles pain, thrombocytopenia, immunosuppression, coagulopathy, and central nervous system manifestations have been largely attributed to the various cytokines released in response to these parasite and red cell membrane products (Clark et al., 2006). Plasmodial DNA is also pro-inflammatory and can induce cytokinemia and fever (Parroche et al., 2007, Schumann, 2007). Hemozoin has also been linked to the induction of apoptosis in developing erythroid cells in the bone marrow, thereby causing anemia (Awandare et al., 2007, Lamikanra et al., 2009).
Malaria infection develops via two phases including exo-erythrocytic and erythrocytic phase. During intake of blood meal, mosquitoes also introduce the sporozoites into blood stream of an individual which migrates to liver and infect hepatocytes. Inside liver cells they multiply asexually and asymptotically for a period of 8–30 days (Bledsoe, 2005).

After this dormant period the parasites are differentiated into thousands of merozoites, rupture the hepatocyte and enters the blood stream to infect the RBCs to start the erythrocytic phase of life cycle (Bledsoe, 2005). During migration from liver cells the parasite wrap itself into membrane of infected host liver cell and become undetected (Vaughan et al., 2008). Within RBCs the parasite multiply asexually and periodically burst the host cell to attack uninfected red blood cells. Several multiplication cycles occur and waves of fever arise from simultaneous merozoites escaping and infecting red blood cells (Bledsoe, 2005).

Some of the sporozoites of *P. vivax* remain dormant instead of developing in merozoites. These dormant sporozoites produce hypnozoites that remain dormant for several months to several years and these hypnozoites can reactivate and develops into merozoites. Hypnozoites are responsible for late relapses in *P. vivax* infections (Richter et al., 2010, White, 2011), while their existence in *P. ovale* is not clear (Richter et al., 2010).

*Plasmodium* parasite is somewhat protected from immune response of the body as it spends most of its human cycle inside liver and blood cells and is relatively undetectable to immune surveillance. However, circulating infected RBCs are destroyed in the spleen. To avoid the destruction of infected RBCs *P. falciparum* parasite displays adhesive proteins on the surface of the infected blood cells which adhere the infected blood cells to the walls of small blood vessels, thereby
sequestering the parasite from passing through general circulation and spleen (Tilley et al., 2011). The blockage of the small vessels causes symptoms such as in placental malaria (Mens et al., 2012). Sequestered red blood cells can breach the blood–brain barrier and cause cerebral malaria (Renia et al., 2012).

1.5.6 Pathogenesis of severe malaria

When malarial parasite (specifically *Plasmodium falciparum*) invade the RBCs, it causes structural, biochemical and mechanical changes of the cell that can worsen if left untreated. *P. falciparum* is reported for most of the severe malaria cases and related mortalities. In recent studies, several cases of severe malaria and even deaths have been reported with *Plasmodium vivax* and *Plasmodium knowlesi* (Price et al., 2007, Tjitra et al., 2008, Kochar et al., 2005, Yale et al., 1993, Cox-Singh et al., 2010, Daneshvar et al., 2009).

In *P. falciparum* infection, the temperature may rise at 48 hour intervals. The ability to cause infected red blood cells (RBCs) to adhere to the linings of small blood vessels is restricted to *P. falciparum*, this sequestration of the parasites cause considerable blockage to blood vessels which may lead to the severity of malaria (Miller et al., 2002).

The basic reason of severe and complicated malaria is the delay in treating an uncomplicated attack of *P. falciparum*. The patient suffers from headache, fever and aches and pains all over the body, diarrhea and sometimes abdominal pain. Spleen and liver are often palpable on clinical examination. The misdiagnosis in non-endemic areas can worsen the clinical symptoms (Schulman et al., 1980).

A patient with severe and complicated malaria will often present with impaired consciousness, weakness, and jaundice. Other complications are cerebral malaria (non-rousable coma), generalized convulsions, normocytic anaemia, renal failure,
hypoglycaemia, fluid, electrolyte and acid-base disturbances, pulmonary edema, circulatory collapse, shock, disseminated intravascular coagulation, hyperpyrexia, hyperparasitaemia, and malarial haemoglobulinuria. These features may occur singly or in combinations (Cheesbrough, 1987).

Multiple pathophysiological factors such as parasite biomass, toxins, inflammatory responses, cytoadherence, sequestration, rosetting, RBC membrane rigidity and deformability, endothelial activation, and altered thrombostasis have been found to be involved in development of severe malaria. All these phenomena are commonly found in *Plasmodium falciparum* as compared to other malarial infections. As a result all severe complications such as hypoglycemia, cerebral malaria, metabolic acidosis, renal failure and respiratory distress with exception of severe anemia are related to *P. falciparum* infection (Chen et al., 2000, Miller et al., 2002).

The development of severe malaria maybe due to combination of parasite-specific factors such as adhesion and sequestration in small vessels and the release of bio-active molecules along with host inflammatory responses. The sequestration of red cells containing mature forms of the parasite (trophozoites and meronts) in the microvasculature is believed to cause the major complications of falciparum malaria, particularly cerebral malaria (Miller et al., 2002).

The sequestration of parasitized red blood cells (PRBCs) in venous beds (relatively hypoxic) allows optimal parasite growth and prevents the PRBCs destruction by spleen. Sequestration is believed to be a specific interaction between PRBCs and the vascular endothelium. Cytoadherence (infected RBCs adhesion to the vascular endothelium) reduces the blood flow in small vessels which may led to organ and tissue dysfunction such as coma. Rosetting (The adherence of non-parasitized red blood cell (NPRBCs) to PRBCs) and agglutination (PRBCs to PRBCs) are also involved in the pathogenesis of
cerebral malaria. Pro-inflammatory cytokines concentration of blood raised in cerebral malaria. Tumor necrosis factor α (TNF-α) up-regulates endothelial cytoadherence receptors and can cause hypoglycemia and dyserythropoiesis (defective development of RBCs), which are features of severe disease (Mohanty et al., 2006, Anstey et al., 2009, Crabb and Cowman, 2002, Garg et al., 1999).

As compare to pathophysiology of *falciparum* malaria there is low knowledge for *vivax* malaria. Because *P. vivax* preferentially infects young RBCs, parasitemia occasionally exceed 2% of circulating RBCs and high parasite burdens are not the characteristics of severe disease. As all stages of *P. vivax* are visible in peripheral blood, *P. vivax* is not supposed to sequester or cause end-organ dysfunction. But, cytokine production during *P. vivax* infections is higher than *P. falciparum* infections of similar parasite biomass (Anstey et al., 2009).

**Figure 1.4 Cytoadherence and rosetting in post-capillary vasculature** (Chen et al., 2000)

1.5.7 **Clinical representation of malaria**

Disease begins only once the asexual parasite multiplies in RBCs. This is the only gateway to disease (Miller et al., 2002).
Most major clinical manifestations of malaria may be attributed to two general factors:

(1) The host inflammatory response, which produces the characteristic chills and fever as well as other related phenomena, and

(2) Anemia, arising from the enormous destruction of red blood cells. Severity of the disease is related to the species producing it: Falciparum malaria is most serious while quartan and ovale are the least dangerous.

The typical presentation of malaria consists of spasms of fever alternating with periods of fatigue but otherwise relative wellness. Before the paroxysm a patient may feel sickness, muscle ache, headache, slight fever, loss of appetite and paroxysm can occur without prior symptoms. Symptoms associated with febrile attacks include headache, high fever, rigors, sweat and as well as muscle pain, backache, abdominal ache, nausea, vomiting, diarrhea, pallor, and jaundice (Robert and Janovy, 2009, Krause, 2007).

Though, classical presentation is seen in only 50%–70% of the cases with the rest having atypical symptoms. In endemic regions, unusual feature of malaria can be seen due to development of immunity, indiscriminate use of antimalarial drugs and the increasing resistance to antimalarial drugs (Singh et al., 1994).

The attack of benign tertian or quartan malaria begins with a feeling of intense cold as the hypothalamus (the body’s thermostat) is activated and there is fast rise in temperature to 104°F to 106°F. Shivering and chattering with nausea and vomiting are common symptoms. The hot stage begins within one half to one hour later, with intense headache and feeling of intense heat. Often a mild delirium stage lasts for several hours. As copious perspiration signals the end of the hot stage, the temperature drops back to normal within two to three hours, and the entire paroxysm is over within 8 to 12 hours. A person may sleep for a while after an episode and feel well until the next paroxysm. Time periods for stages are usually somewhat shorter in quartan malaria, and
paroxysms recur every 72 hours. In vivax malaria periodicity is often quotidian early in the infection because two populations of merozoites usually mature on alternate days. “Double” and “triple” quartan infections also are known. Only after one or more groups drop out does fever become tertian or quartan, and a patient experiences the classical good and bad days (Bruce-Chwat, 1980, Robert and Janovy, 2009).

Generalized seizures are associated specifically with falciparum malaria and might be followed by coma (cerebral malaria). Most patients with uncomplicated infections have few abnormal physical findings other than fever, mild anemia, and, after several days, a palpable spleen. The liver can become enlarged, especially in young children, whereas mild jaundice is more likely in adults. Literature on the hepatic involvement in malaria has largely shown severe infection with *P. falciparum* infection. There have been occasional reports of mixed infection with *P. vivax* (Joshi et al., 1986) and hepatitis E (Bansal et al., 2002) along with *P. falciparum*, resulting in malarial hepatitis.

In young children living in regions in which transmission is stable, recurrent infections cause chronic anemia and splenomegaly. The manifestations of severe falciparum malaria, depend on age (Cordery and Urban, 2009). Severe anemia and hypoglycemia are more common in children, whereas acute pulmonary edema, acute kidney injury, and jaundice are more common in adults; coma (cerebral malaria) and acidosis occur in all age groups. Mortality rises when the proportion of infected erythrocytes (parasitemia) exceeds 2%, although the relation between parasite density and prognosis in falciparum malaria is very variable. When treated promptly with effective antimalarial drugs, uncomplicated falciparum malaria has a mortality of roughly 0.1%. The main causes of the anemia are destruction of both infected and non-infected erythrocytes, inability of the body to recycle the iron bound in hemozoin and an insufficient erythropoietic response of the bone marrow. Why such large numbers of
non-parasitized red cells are destroyed is still not understood, but some evidence has indicated complement mediated, autoimmune hemolysis. In acute malaria, the spleen removes substantial numbers of non-parasitized red cells from the blood, an effect that may persist beyond the time of parasite clearance (Chang and Stevenson, 2004). Both the splenic removal of red cells and the defective bone marrow response may be due in part to TNF toxicity (Clark, 1987, Mendis and Carter, 1995). Destruction of RBCs leads to an increase in blood bilirubin (breakdown product of hemoglobin). Excessive accumulation of bilirubin cause jaundice (yellows the skin). Circulating leukocytes taken up the hemozoin and deposited in the reticulo-endothelial system. In severe cases the viscera (especially the liver, spleen, and brain) become blackish or slaty as the result of pigment deposition. Ingestion of hemozoin impair the phagocytic ability of macrophages (Turrini et al., 1993).

Hypoglycemia (reduced concentration of blood glucose) is a common symptom in *falciparum* malaria. It is usually found in women with uncomplicated or severe malaria who are pregnant or have recently delivered as well as in other cases of severe *falciparum* malaria (WHO, 1999, Clark, 1987). Coma produced by hypoglycemia has commonly been misdiagnosed as cerebral malaria. This condition is usually associated with quinine treatment. Pancreatic islet cells are stimulated by quinine to increase insulin secretion, thus lowering blood glucose and this effect may also be due to excessive TNF (Clark, 1987).
1.6 Recurrent or persistent malaria

The blood stage of *Plasmodium* infection can persist for a long period (months or years or even decades in *P. malariae* infection). In tropical areas, the *Plasmodium vivax* relapse occurs every 3-4 weeks or every 6-8 weeks in case of treatment with slowly eliminated drugs that suppress the 1st relapse). Blood-stage infection can persist for months or years (or decades in *P. malariae* infections) when untreated. Waves of asexual parasitaemia and gametocytaemia result from antigenic variation. In tropical regions, *P. vivax* relapses typically every 3–4 weeks (or every 6–8 weeks after treatment with slowly eliminated drugs, which suppress the first relapse). In temperate areas, *P. vivax* can remain latent for 8–10 months between primary infection and first relapse (Simpson et al., 2002). Recurrent *falciparum* and *vivax* malaria have pronounced
adverse effects in young children, and interfere with growth, development, and schooling.

1.7 Immunity against parasite

Partial immunity based on humoral antibodies that block merozoites from invading the red cells occurs in infected individuals. A low level of parasitemia and low grade symptoms results; this condition is known as premunition. In contrast, a non-immune person, such as a first-time traveler to an area where falciparum malaria is endemic, is at risk severe, life-threatening disease (Levinson, 2014).

Cell-mediated and humoral immunity play active roles in immune-competent individuals living in malaria endemic areas. More immunoglobins especially IgG is raised when the immune cells are activated by specific antigens, for instance, circumsporozoite proteins found on sporozoites. Vaccines are currently being developed against three stages of the parasite: gametocytes, sporozoites, and intra-erythrocytic merozoites (Vanderberg, 1980, Rogerson et al., 2007). The most important part of host defense seems to be antibody production; hence non-immune individuals visiting endemic areas are very vulnerable.

1.8 Treatment and Prevention of Malaria

1.8.1 Treatment

Extracts of bark from Peruvian trees were used with varying success to treat malaria, but alkaloids from the bark of certain species of Cinchona proved dependable and effective (Honigsbaum, 2001, Robert and Janovy, 2009). The most widely used of these alkaloids was quinine, discovered in the 16th century. The alkaloid of D. febrifuga, febrifugine, is now considered too toxic for human use, but the terpene from A. annua, called qinghaosu (artemisinin), which has recently been “rediscovered,” and its derivatives are valuable drugs (Robert and Janovy, 2009). P. vivax infection is
commonly treated with chloroquine, but parasites are often treated with sulphadoxine-pyrimethamine, the commonly used drug for chemoprophylaxis and treating uncomplicated falciparum malaria (WHO, 2017).

The most important drug was chloroquine but due to emergence of chloroquine resistance new drugs were developed including primaquine, mefloquine, pyrimethamine, proguanil, sulfonamides such as sulfadoxine, and antibiotics such as tetracycline. Among all these only Primaquine is effective against all stages of all species as the other drugs vary in efficacy according to species and stages of parasites. Primaquine and chloroquine drugs of choice for *Plasmodium vivax* and *Plasmodium ovale* malarias and chloroquine alone for *Plasmodium malariae* infections. Chloroquine is still advised for *P. falciparum* strains which are sensitive to this drug (Geary and Jensen, 1986). *P. falciparum* chloroquine resistant strains has now spread through Africa, Asia, and South America (D’Alessandro and Buttiens, 2001) and resistance to other drugs also persist. Sulfadoxine and pyrimethamine (Fansidar) combination was used for chloroquine-resistant falciparum malaria, but Fansidar-resistant *P. falciparum* is now present in many regions. Mefloquine (Lariam) is still effective for multidrug-resistant *P. falciparum*, but resistance to mefloquine is developed in several endemic areas (Mockenhaupt, 1995). Artemisinin and its derivatives are effective for drug-resistant *P. falciparum*, both in severe and uncomplicated malaria (Sowunmi and Oduola, 1996). The artemisinin derivative is commonly given in combination with other drugs (Artemisinin-based combination therapies, ACTs) (D’Alessandro and Buttiens, 2001). Dihydroartemisin with Piperaquine (Artekin) is an ACT with the advantage that it is also low cost (Mutabingwa, 2005).
1.8.2 Prevention

There are two ways to prevent malaria. One is vector control and other is use of antimalarial drugs. The main approach for preventing and decreasing malaria transmission is the vector control. WHO recommended the protection against malaria with effective vector control measures for all people who are at risk. Insecticide treated nets and indoor residual spraying are the most effective way for vector control. The long-Lasting insecticidal nets are recommended by WHO in most of the settings (WHO, 2017). For effective vector control it is necessary to spread awareness among individuals, use of efficient insecticide usage and destruction or elimination of vector breeding sites.

The use of prophylactic drugs has been generally effective for travelers and people living in endemic areas. Several aspects should be kept in mind while prescribing chemoprophylactic drugs, such as parasite species, duration of travel, parasite transmission intensity in the specific area and existing antimalarial drug resistant strains in the area. It is very hard to recommend prophylactic drugs for South East Asia due to the high degree of resistance there (Bradley, 2001). The use of antimalarial drugs can also be useful to prevent malaria. Before travelling the chemoprophylaxis is helpful in suppressing the blood stages of *Plasmodium*. In moderate to high transmission areas, WHO recommends preventive treatment for pregnant females with sulfadoxine-pyrimethamine at each scheduled antenatal visit after the 1st trimester. Similarly, in high transmission areas three doses of intermittent treatment with sulfadoxine-pyrimethamine are recommended for infants, delivered alongside routine vaccinations (WHO, 2017).
In areas of low endemicity especially in non-African regions the human-mosquito contact can be reduced by appropriate construction and siting of housing and local environmental improvement. Inexpensive, high-technology approaches will apply computerized information management and Geographic Information Systems to identify locations where targeted attack on malaria transmission is called for. Monitoring, including by satellite, of all aspects and features of a malarious situation will be important to the timing and targeting of antimalarial interventions (Carter et al., 2000, Thomson and Connor, 2001).

The management of malaria also aim to reduce the number of individuals at risk of malarial infection and to reduce human mosquito contact by all possible means. These means will certainly include, especially in Africa, provision and use of insecticide-treated materials such as bed nets and curtains (Guillet et al., 2001, N’Guessan et al., 2001, Lengeler, 2000) for those at highest risk mainly infants, young children and pregnant women.

1.9 Malaria vaccines

RTS,S/AS01 (RTS,S) known as Mosquirix is an injectable vaccine that provide partial immunity against malaria in childrens. This vaccine is evaluated in sub Saharan Africa as a complementary malaria control tool. In 2015, a positive opinion was given in favor of this vaccine by European medicine Agency (Medicine regulatory Authority). In the same year WHO recommended this vaccine to be implemented in a limited number of African countries. In 2016, WHO announced pilot project to be implemented in three countries of sub-Saharan Africa for which funding have been secured. This project would be further implemented on broad scale on the basis of success of pilot projects. (WHO, 2017).
1.10 *Antimalarial drug resistance*

In Pakistan, chloroquine resistance was first reported in the mid-1980s, which spread throughout the country (Shah et al., 1997, Rowland et al., 1997a). During the last decades, Chloroquine resistance is the key reason for high prevalence of *P. falciparum* malaria in Pakistan (Shah et al., 1997), Afghanistan (Rab et al., 2001), and neighboring Tajikistan (Pitt et al., 1998), whereas conflict and population disturbance are the factors responsible for the overall upsurge in malaria in the region (Kazmi and Pandit, 2001).

In previous studies, it was found that about 90% of the *P. falciparum* cases among Afghan refugees in western Pakistan were sensitive to sulfadoxine-pyrimethamine (Rowland et al., 1997b) & *P. vivax* malaria is still sensitive to chloroquine in this population (Rowland et al., 1999).

*P. falciparum* has acquired resistance to earlier generations of antimalarial drugs (chloroquine and sulfadoxine-pyrimethamine) thus has led to increased child mortality and destability of the malaria control programs. WHO encourages the routine monitoring of antimalarial drug resistance and recommends countries to enhance their efforts in this regard. An artemisinin-based combination therapy (ACT) contains both the drug artemisinin and a partner drug. Recently the *Plasmodium* resistance to artemisinin has been detected in five countries of the Greater Mekong sub-region: Cambodia, Myanmar, Vietnam, Thailand and Lao People’s Democratic Republic.

Studies have confirmed that artemisinin resistance has emerged independently in many areas of this sub-region. In 2013, Emergency response to artemisinin resistance (ERAR) was launched by WHO in the greater Mekong region but when this project was underway, another independently emerged spots were reported in the same sub-region. Similarly, increased resistance to ACT partner drug was also reported in the same
locales. Thus a new approach is required to cope with changing malaria scenario (WHO, 2017).

1.11 Worldwide malaria epidemiology

Malaria is a major public health problem, and is endemic in approximately 130 countries and territories including those countries that have not reported malaria recently, but they reported cases in the period 1990-2003 (WHO, 2009). The global burden of malaria is only imprecisely known, because only a minority of cases are recorded in health facilities, yet vital registration of death causes is incomplete and unreliable in countries with highest malaria burden and available community-based studies often over sample areas of relatively intense malaria transmission, usually conducted during peak transmission season (Snow et al., 2005, Korenromp, 2005).

Malaria is the leading cause of mortality in children (< years age) and pregnant women in developing countries (Martens and Hall, 2000, Lagerberg, 2008). In 2010 there were an estimated 216 million cases of malaria worldwide, of which 91% were due to P. falciparum. The large number of cases (81%) were in the African Region followed by South-East Asia (13%) and Eastern Mediterranean Regions (5%) (WHO, 2011a). The disease remains one of the most important cause of human morbidity and mortality with enormous medical, economic and emotional impact in the world.

Globally the number of malaria cases are increasing due to increasing transmission risk in areas where malaria control has declined, increasing frequency of drug resistant strains of parasite and in a comparatively few cases, considerable increases in international travel and migration (Pasvol, 2005). In 2013, 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). Most of the malaria cases (80%) and deaths (90%) occurred in Africa and most deaths (77%) were in children under 5 years of age (WHO, 2014a).

According to recent reports by World Health Organization (WHO) there are about 438,000 people died because of malaria in 2015 while the Institute of Health Metrics and Evaluation (IHME) and Global Burden of Disease (GBD) puts this estimate at 720,000. The major victims are children and about 72% mortality was reported in children under 5 years of age (WHO, 2017).

Figure 1.6 Estimated country share of (a) total malaria cases and (b) P. vivax malaria cases, 2016 (WHO, 2017)
About 216 million malaria cases were reported to occur worldwide in comparison with 211 million cases in 2015 and 273 million cases in 2010. These figures shown the reduction in global malaria prevalence. The WHO African region (90%) is reported with most of the cases which is followed by South-East Asia region (7%) and Eastern Mediterranean region (2%). Malaria incidence rate was estimated to have decreased by 18% globally, show reduction from 76 to 63 cases per 1000 population (at risk) from 2010 to 2016. Largest decline was documented from WHO South-East Asia region (48%) followed by Americas (22%) and African region (20%) (WHO, 2017).

*Plasmodium falciparum* was found most prevalent in sub-Saharan Africa, responsible for 99% of estimated malaria cases in 2016. *P. vivax* is predominant species outside Africa and responsible for 64% cases in WHO Americas region, above 30% in the WHO South-east Asia and 40% in the Eastern Mediterranean regions (WHO, 2017). In 2016, about 445,000 deaths were reported globally while 446,000 estimated cases in 2015. Reduction in mortality was recorded in 2016 when compared to 2010, with exception of WHO Eastern Mediterranean where mortality is remain unchanged in that period. While largest reduction was documented in WHO regions of South-East regions (44%), Africa (37%) and the America (27%) (WHO, 2017). Many countries are moving towards malaria elimination: 44 countries reported fewer than 10, 000 malaria cases (2016) and 37 countries in 2010. WHO certified Kyrgyzstan and Sri Lanka as malaria free in 2016 while 21 countries were identified with elimination potential by the year 2020. WHO is working in collaboration with government of these countries which were termed as “E-2020 countries” to support their elimination efforts (WHO, 2017).
In 1955 the World Health organization started the efforts to eradicate endemic malaria which included the spraying of houses with residual insecticides, anti-malarial drug treatment, and continual surveillance (CDC, 2017b). Successful elimination was done in countries (including Europe and Australia) with temperate climate and seasonal transmission of malaria. Many other countries (India and Sri Lanka) initially showed marked reduction in cases, however these and other countries including all of sub-Saharan Africa, had little long term success. Many factors were involved in failure of long term elimination including lack of funds, wars and population disturbance due to wars, emergence of drug resistant strains and arrival of insecticide resistant vector (WHO, 2009).
1.12 Malaria epidemiology in Pakistan

Pakistan shares the highest burden of major vector-borne diseases as malaria, leishmaniasis, dengue, Chikungunya and Crimean-Congo haemorrhagic fever. One of the leading cause of mortality and morbidity in Pakistan is malaria. Malaria in Pakistan remains the fourth largest cause of death among communicable diseases. WHO (2006) places Pakistan in category 3 countries (with moderate endemicity) in the eastern Mediterranean Region. During 2006, a total of 127,825 microscopically confirmed cases were reported from public sector malaria microscopy centers all over the country. The overall API (annual parasite index) of Sindh, Baluchistan, and FATA and KPK provinces in 2006 was 4.0 per thousand population. However, the actual API may be 5 times higher than the reported figures, as the public sector FLCFs report only 20% of malaria cases. HMIS report 2006 shows malaria as the 2nd commonest illness after ARI. HMIS reported 4.3 million clinical and confirmed cases treated at 10,000 public sector health outlets. The slide positivity rate (total number of positive slides per 100 slides examined) for malarial parasite was highest in Baluchistan than other provinces. More than 90% of disease burden in the country is shared by the 56 highly endemic districts, mostly located along the international borders with Iran and Afghanistan. More than 40% of the reported cases from these districts are due to *P. falciparum* malaria (DMOC, 2012).

Pakistan has a population of 180 million inhabitants of which 177 million are at risk of malaria with 3.5 million presumed and confirmed malaria cases annually. Among the reported cases 37% of malaria are from the districts and agencies of Federally Administered Tribal Areas (FATA) and Baluchistan bordering Afghanistan and Iran. (DMC, 2017).
Plasmodium vivax and Plasmodium falciparum are the only prevalent species of parasites detected so far, with P. vivax being the major parasite species responsible for >80% reported confirmed cases in the country (DMC, 2017, WHO, 2011a, Ghanchi et al., 2010). Malaria transmission pattern is unstable and seasonal, with peaks in summer (June-September) for vivax malaria and late-summer and winter (August- November) for falciparum malaria (DMC, 2017, Gill, 1938, Rowland et al., 1997a) (Macdonald, 1956, Bouma et al., 1996a). Most of the spring cases remain dormant in the liver and appear after the monsoon season especially P. vivax infection (MOH, 2010, Asif, 2008, Khan et al., 2006). At the northern part of Pakistan, P. falciparum showed unstable pattern and prominent fluctuations from year to year depending on climatic variations. High rainfall in autumn or raise of temperature than average range in November & December are the significant factors that contributes to extend the transmission period in Pakistan (Bouma et al., 1996a).

The major epidemics of malaria in Pakistan was recorded before Pakistani independence in 1908, which was the most devastating outbreak with over 300,000 deaths were documented in an estimated population of 20 million while last epidemic was reported in the mid-1970s (de Zulueta et al., 1980).

The estimated burden of malaria in Pakistan is 1.6 million cases per year, grouped into Group-3 countries of Eastern Mediterranean (countries with highest malaria burden) along with Afghanistan, Sudan and Yemen. 98% of the malaria cases were reported to be contributed by these four countries in this region (WHO, 2017). Huge population movements within the Pakistan and across international borders (Iran and Afghanistan), natural catastrophes and civil unrest, unpredictable transmission pattern due to climate changes, poor socio economic status, declining health infrastructure due to internally displaced population, resource limitations, limited access to health facilities (preventive
and curative services) and lack of monitoring drug resistance in parasites and insecticide resistance in vectors has added in making malaria a serious public health problem. Malaria is usually unstable in Pakistan and all age groups are prone to acquire infection. However, children (5-year age) and pregnant women are more susceptible to malaria (Directorate of malaria control, 2014). In Pakistan, among 24 species and sub species of Anopheles, only two are the primary vectors involved in malaria transmission (Castro et al., 2010, Alemu et al., 2012). Earlier studies reported the A. culicifacies as the important vector in rural settings (mehmood & MacDonald, 1985; Pervez & Shah, 1989) while A. stephensi in the urban areas (Rehman and Muttalib, 1967). Both primary vectors are endophilic in diurnal resting habit, breed in clean water and chiefly zoophilic.

Malaria control program is implemented in 72 malaria endemic districts by Government of Pakistan while in 19 highly endemic districts were supported from Global Fund. The highly endemic districts are the main target for malaria control program and target population in these 38 districts is 28.84 million individuals. The aim of malaria control program is to reduce the disease burden in these high risk districts by elevation appropriate intervention including improving case management, treating all confirmed Plasmodium infected cases according to national guidelines and vector control through IRS and LLINs (Directorate of malaria control, 2014, DMC, 2017).
In Pakistan, Afghan refugees are at high risk of malaria infection rather than that they brought a high infection load with them from Afghanistan. Therefore, malaria should be controlled on preventive base in Afghan refugees’ camps in Pakistan (Suleman, 1988a).
Factors associated with the upsurge include of chloroquine resistance across the country (Shah et al., 1997), warmer autumns favoring prolonged transmission (Bouma et al., 1996b, Shah et al., 1997) and a chronic decline in vector control activities.

*Anopheles culicifacies*, the purported primary vector in the Punjab Province (Reisen and Boreham, 1982) was found disappeared by September whereas *A. stephensi* was found more abundant and more common in KPK Province than *A. culicifacies*. In Pakistan, the primary vector species are *A. culicifacies* and *A. stephensi* and in Quetta Balochistan also (Yasinzai and Kakarsulemankhel, 2003, Yasinzai and Kakarsulemankhel, 2004).

In 2016, 3.1 million cases were diagnosed in public sector facilities. The malaria indictor survey (MIS) was conducted in 2013 in thirty-eight endemic districts of Pakistan. Federally Administered Tribal Areas (FATA) presented highest prevalence rates (13.9%) (DMC, 2017). While in 2015 high Annual parasite incidence (8.60/1000 Population) in FATA was reported which is followed by (5.52/1000 Population) in KP, (4.47/1000 Population) in Baluchistan and (2.11/1000 Population) in Sindh (DMC, 2017).

Based on the reported API, FATA followed by Baluchistan and Khyber Pakhtunkhwa are the highest endemic provinces/regions. According to the latest “Stratification” 66 districts and agencies have been categorized in “high endemicity stratum” where API is >5/1000 population (WHO, 2017).

In the last decade, there has been a six-fold increase in *Falciparum* malaria which comprised 42% of all malaria cases recorded by National Malaria Control Program (Shah et al., 1997) Many factors are involved in increase of malaria cases such as warm autumns favoring prolonged transmission (Bouma et al., 1996a) emergence of chloroquine resistance across the country (Shah et al., 1997) and a chronic dropping of
vector control activities. Migrated population can play a vital role in malaria transmission in various ways (Rowland et al., 2002). An increasing population in malarious regions, compounded by weak public health systems in developing countries, climate changes, new agriculture practices such as irrigation and dam construction (Sachs and Malaney, 2002), increased resistance to antimalarial treatments and insecticides (Bozdech et al., 2003) and the complexity and flexibility of the genetics (Gardner et al., 2002) have all contributed to increase in malaria (Eze and Mazeli, 2001).

In KP malaria (particularly *P. falciparum* malaria) is unstable and fluctuates from year to year due to climatic variations and malaria outbreaks as reported in past (Bouma et al., 1996a) *P. falciparum* transmission season starts in the summer monsoon (July) when the temperature and humidity is suitable and remains until the end of the year when the temperature falls below the critical value (December). *P. vivax* has usually two peaks of transmission in the year, one is its early transmission period during the wet months of spring due to its lower temperature requirement (probably facilitated by true relapses) and the other is with the *P. falciparum* (monsoon) (Bouma et al., 1996a).

Malaria remains a serious health problem in all districts of KP but two geographical clusters including one in the North East (Malakand, Buner, Shangla, and Dir) and the other in the South West (Kohat, Lakki Marwat, Hangu, Bannu, Tank, Dera Ismail Khan) were recorded as high risk zone of malaria (Asif, 2008). In district Bannu the annual parasite incidence (API) is 1.6 - 3.5 per 1000 population, which is substantially above the national average (0.8 per 1,000 population). The district has great economic significance, being the central market of the Southern Region in addition to serving as a safe shortcut to markets in Central Asia (Bouma et al., 1996a, MOH, 2010). The *falciparum* malaria having fatal complications is reported as serious illness in southern
and northern districts of KP and delay in treating *falciparum* malaria may leads to serious consequences (Raziq and Khan, 1995).

### 1.13 Diagnosis

Accurate and early detection of *Plasmodium* infection is necessary for effective management and malaria surveillance. To avoid morbidity and mortality from malaria, the availability of standard malaria diagnostics is essential in all settings.

#### 1.13.1 MICROSCOPY

Microscopic detection of malaria parasite from blood smear is used as a gold standard for any new malaria diagnostic tool as it can detect and differentiate each species of *Plasmodium* (Alam et al., 2011, Batwala et al., 2011, Ouattara et al., 2011, Tangpukdee et al., 2009, Wongsrichanalai et al., 2007). However, microscopy have several drawbacks such as requiring a visual or light microscope with 1000× magnification and depends on expert and well-trained microscopists. Microscopic detection of malaria parasite is morphology based method so there are chances of incorrect identification of morphologically close species of *Plasmodium* such as *P. knowlesi* and *P. malariae*, even by an expert microscopists. To avoid human errors, an image processing technique is recently introduced for microscopy. Still the problem of detecting low parasitemia by microscopy is not resolved (Das et al., 2015, Tek et al., 2009). This problem is due to the average ability of parasite detection of microscope which is around 10 parasite/µL for a research setting and in the range of 50–100 parasites/µL for outside a research setting or less sensitive in inadequate resource setting (Payne, 1988).

Fluctuation in parasite density during infection limits the detection power of microscope and all other direct diagnostic approaches (Hawkins et al., 2014). In remote
rural settings, the lack of microscopy might be resolved in this near future due to the
development of an origami-based paper microscope (Cybulski et al., 2014). This device
was deliberately developed for malaria control in a poor country.

For microscopy Patient’s blood drop collected by finger pricking is spread on glass
slide to make a smear. Smear is dipped in a dilute Giemsa stain solution (for staining
the malarial parasite) and examined under microscope at 1000x magnification.
Morphology of altered RBCs (altered by parasite) and physical features of malarial
parasite are used for diagnosis of malarial infection. It is a simple and established
technique that is familiar for most laboratorians. Correct and confirmed laboratory
diagnosis of malaria needs complete knowledge about morphological features of
different blood stages of all plasmodia species. For correct diagnosis, careful
observation of different morphological features that draw a well-defined image of the
species and at least 100 microscopic fields observation should be done before
classifying the slide as negative. A single image of parasite does not reflect the real
cause of infection as the possibility of mixed infection can’t be rule out for example a
crescent-shaped gametocyte in peripheral blood does not mean that a *P. falciparum*
infection is the cause of the clinical complaint as the possibility of mixed
infection always exist (PARN, 2016).

Microscopy is the most widely tool used to diagnose malaria at peripheral levels. In
capable hands, it is very sensitive for parasitemia ≤50/μL (0.001%) (Nandwani et al.,
2005) and it can give information of species, parasites stages and parasite density.
However, implementation of good quality of microscopy is difficult. As it is labor
intensive, requires highly skilled personnel and regular quality control. Despite of all
these shortcomings it is still a gold standard for malaria diagnosis (Nandwani et al., 2005).

Currently, microscopic examination of thick and thin blood smear is the conventional method for detecting acute malaria. Although it is cheap, convenient, rapid, inexpensive and relatively accurate diagnostic method but requires highly skilled technician, labor intensive and not sensitive enough to detect low parasitemia level. Therefore, this technique is inappropriate for large scale epidemiological studies. Sensitivity limit of the thick smear examination is estimated at ~5 parasites/μL (100 microscope field’s examination) (Barker et al., 1992). Parasite densities of 4-40 parasites/μL (1/200 to 1/2,000 parasites per leukocytes) is rarely detected (Gilles, 1933) and in busy, routine examination, the sensitivity level is 10 time lesser and ratio of false negative detection in oligo-parasitemic patients and false positive detections due to artifacts can’t be neglected (Grant et al., 1960). Keeping in mind these limitations, there is need to develop alternative malaria diagnostic techniques. Alternative method to microscopy is immunologic test which is also less sensitive and cannot differentiate past and present infection. Concentration of malaria parasite–infected red blood cells by centrifugation coupled with Acridine orange staining and fluorescence microscopy (quantitative buffy coat (QBC) System, Becton Dickinson) is easier to use and more sensitive (Rickman et al., 1989), but its cost and need for special equipment (Centrifuge, Fluorescence microscope and capillary tubes) is its main limitations (Avila and Ferreira, 1996). DNA hybridization methods show a modest improvement in sensitivity compared with blood films (Waters and McCutchan, 1989). To minimize the risk of malaria transmission more sensitive technique is required to replace microscopy.
### 1.13.2 Rapid diagnostic Technique (RDT)

Early diagnosis and prompt treatment is one of the key strategies in controlling malaria. For areas where laboratory facilities are not available, clinical diagnosis is widely used (WHO, 1999). Due to difficulties in maintaining standard microscopy in peripheral health facilities, the introduction of rapid diagnostic tests (RDT) for malaria is a major step for case detection, management and reduction of unnecessary treatment. RDT could be used in malaria diagnosis during population-based surveys and to provide immediate treatment based on the results (WHO, 2009). These tests use a drop of blood from finger, take only 10 to 15 minutes to complete and do not require a laboratory. Non-clinical staff can easily learn to perform the test and interpret the results (Wongsrichanalai et al., 2007).

The test is based on the detection of parasite antigen from the peripheral blood using monoclonal antibodies (against malaria antigen target) and conjugated to either a gold particles or liposome containing selenium dye in a mobile phase. Or reversed where instead of monoclonal antibody to capture the antigen, antigen is incorporated into the cellulose to capture the antibody in the serum or plasma (Azikiwe et al., 2012).

RDTs commonly available in two forms. One is antigen based and normally requires the use of haemolyzed red blood cells while the other is antibody based and normally requires the use of extracted serum. Antibodies are better expressed in serum otherwise plasma could also stand in place of serum for antibody based method (Moody, 2002). Rapid diagnostic technique is expected to play significant role in malaria control. It immunologically detects different malaria antigens such as Histidine-rich protein-2 (HRP-2), lactate dehydrogenase (LDH) and Aldolase and in a few microliter of blood (typically 5–15 μL) (Piper et al., 1996, Oner et al., 2004). The RDT strip has been extensively tested for its impacts on malaria diagnosis outside research settings for the
past few years (Sudhinaraset et al., 2015, Hansen et al., 2015, Boyce et al., 2015), where use of RDTs increased the proportion of patients with a parasite-based diagnosis of malaria compared to microscopy alone, leading to a higher accuracy, timely clinical case management and better cost effectiveness (Boyce et al., 2015, Hansen et al., 2015). Some RDTs can detect a single species (either *P. falciparum* or *P. vivax*) while others can detect multiple species (*P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*) (Foster et al., 2014). Although this technique is timely and easy to use, it is relatively expensive and subjected to false-positive responses due to the persistence of malaria antigens in the blood for up to 2 weeks after the parasite clearance from the patient’s circulation. In addition, limits of detection (LOD) of these RDTs rely on an amount of antigen (equivalent to 200 infected RBCs/µL or 2000–5000 parasites/µL of blood) (WHO, 2014a).

HRP-2 based tests may be misleading in areas of high transmission because they remain positive for several days or weeks after an infection, even if treated, thus a positive result with a history of a recently treated infection is difficult to interpret. Another limitation of HRP-2 based tests is their geographically variable sensitivity, attributed to polymorphisms in HRP-2 (Baker et al., 2005). Tests based on detection of pLDH or aldolase allow parasite speciation, do not appear to show geographical variability in their ability to detect malaria and revert to negative more quickly than HRP2 based tests, although production of pLDH from gametocytes after elimination of asexual stages means some will stay positive for several days (Tjitra et al., 2001, Pattanasin et al., 2003, van den Broek et al., 2006). However, to date the sensitivity of these tests under field conditions has been reported frequently as falling below 90% (Pattanasin et al., 2003). There are concerns about the stability of all types of tests if transportation
and storage conditions are not controlled, but pLDH tests appear to be particularly vulnerable (Jorgensen et al., 2006).

Figure 1.10 Mechanism of action of Antigen-detecting malaria RDT (WHO, 2011a)

1.13.3 Polymerase chain reaction (PCR)

Molecular techniques such as Polymerase Chain Reaction (PCR) and Nucleic Acid Sequence-Based Amplification (NASBA) have been proposed since 1990 for malaria diagnosis by using various kinds of primers, DNA extraction and detection methods. PCR is said to detect less than 5 parasites per µl of blood (Snounou et al., 1993b). PCR is more sensitive and specific than all other techniques. However, it is a lengthy procedure that requires specialized and costly equipment and reagents as well as laboratory conditions that are often not available in the field (Snounou et al., 1993a). It is the most sensitive and specific technique yet measured as a lengthy procedure with
advanced and expensive equipment not favorable for clinical use. Laboratory conditions are also not applicable in the field (Hanscheid and Grobusch, 2002). PCR is a scientific method for amplification of a single or multiple genes to generate thousand to million copies of a DNA sequences. It is common and essential technique used. There are three major steps involved in the PCR technique: denaturation, annealing, and extension (Joshi and Deshpande, 2010).

The low level parasitaemia (in case of asymptomatic individual) is reported with significant contribution in malaria transmission (Shekalaghe et al., 2009). Due to sub-microscopic infection, the estimated cases of malaria infection detected through microscope was found lower than actual number of cases existing in the population. To reduce the parasite transmission, it is essential to quantify sub-microscopic cases in a population (Okell et al., 2009). Many studies documented the detection of low density infection through PCR due to its higher sensitivity for low level infection as compare to microscopy (Okell et al., 2009, Snounou et al., 1993b, Musalika, 2010a).

Due to high sensitivity and species level identification, PCR is used to compare the sensitivity and specificity of other Plasmodium detection techniques (Mikhail et al., 2011). Presently PCR is not used as routine diagnostic approach due to its time consumption and technical expertise requirements. The major drawback of PCR based detection is the expensiveness as compared to microscopy, RDT and serology (Wahid, 2013).

Most of the PCR assays used for diagnostic purpose targets the genes 18S subunit rRNA gene or single stranded rRNA. (Gunderson et al., 1987, Kawamoto et al., 1996, Singh et al., 1999a, Snounou et al., 1993a). Amplification of these genes is useful for detecting Plasmodium species in case of low parasitaemia or clinical samples (filter paper or from slide) which have low parasite DNA content. The basic disadvantage of PCR is the
contamination and specificity as it need very specific conditions and slight variation can lead to dramatic reduction in PCR products (Coleman et al., 2006b).

Real time PCR is new advancement in molecular techniques serving as most sensitive and time efficient assay (Lee et al., 2002). It can detect 0.01 to 0.02% level of parasitemia, differentiate species and recognize mixed infection (Mangold et al., 2005). It is economical in term of time as single probe is used with results based on melting curve analysis instead of gel based analysis (Boonma et al., 2007). However, it is also very expensive method and needs skill and experience, (Brown et al., 1979, Mangold et al., 2005). Most of the real-time PCR assays target the SU 18s RNA (Andrews et al., 2005, Elsayed et al., 2006, Hermsen et al., 2001, Vo et al., 2007). The main drawback of real time PCR is use of expensive consumables. However, duplex real-time PCR is cost effective up to as it can detect *P. falciparum* and *P. vivax* in the same tube (Veron et al., 2009).

The conventional nested PCRs are highly sensitive and specific. Commonly used diagnostic method developed by Snounou and colleagues uses the SU 18s rRNA nested primers that differentiate between four *Plasmodium* species i.e. *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae* and *Plasmodium ovale* (Snounou et al., 1993a). However, nested PCRs have long running time and there is high risk of contamination while Real-time PCR have the advantage of low contamination chances due to absence of post-PCR handling. Loop-mediated isothermal amplification (LAMP) is a simple, rapid and sensitive method with high specificity. LAMP uses Bst DNA polymerase and a set of four primers that bind to six distinct regions of the target gene that produce a specific double hairpin DNA template. The target gene is amplified isothermally for about one hour. The amplified products can be achieved, either by visually comparing the turbidity of magnesium pyrophosphate (a byproduct of DNA
synthesis) or by real-time detection through real-time turbidimeter (Notomi et al., 2000, Nagamine et al., 2001, Mori et al., 2001).

LAMP is found more suitable in field settings for malaria diagnosis as the conventional microscopy suffer from low sensitivity in case of low parasitaemia and inexperienced personnel (Sirichaisinthop et al., 2011). The specificity and sensitivity of this method is shown to be comparable with that of nested PCR (Han et al., 2007). However, previous studies report the false positive and false negative results by this method. LAMP is prone to errors for visual of interpretation of turbidity to test positivity are subject to individual variation in interpretation (Paris et al., 2007).

PCR-based malaria diagnosis is the most efficient technique which have high specificity and sensitivity in species level detection. PCR method can be categorized into nested PCR, semi-nested PCR, single step multiplex PCR and real-time or quantitative PCR assays (Ongagna-Yhombi et al., 2013, Lee et al., 2015). Among them, the simplest and least technically demanding is a loop-mediated isothermal amplification (LAMP) assay (Cook et al., 2015a). Usually, PCR can detect low parasitemia (5 parasites/μL) for all human malaria parasites (Moody and Chiodini, 2002, Cook et al., 2015b). Tests based on polymerase chain reaction for species-specific Plasmodium genome are more sensitive and specific than are other tests, detecting as few as 10 parasites/μl blood (Hanscheid and Grobusch, 2002). It is recently reported that saliva, fecal and urine samples of Plasmodium vivax and Plasmodium falciparum infected patients contain Plasmodial DNA that can be amplified through PCR (Putaporntip et al., 2011, Jirku et al., 2012).

PCR efficiency depends on the complimentary nucleotide sequences between the primer (known sequence) and its counterpart target DNA (unknown sequence), thus parasites with genetically modified sequences at primer’s target region are subjected to
detection failure or to a lower amplification efficiency that ultimately reduce the sensitivity of PCR. Additionally, the detection power of PCR can be effected by copy number of target gene or nucleic acid sequence in the *Plasmodium* genome. Small subunit 18S ribosomal RNA gene (18S rDNA) is the most commonly used target in PCR (Kawamoto et al., 1996). This gene is highly conserved across *Plasmodium* species but to limited number of parasite in the infected cells the amplification of mitochondrial DNA is preferred for parasite detection. As mitochondrial DNA is abundant in parasite (30-100 copies per parasite) than nuclear DNA.

Another favorable target is the mitochondrial DNA due to its more abundance (30-100 copies per parasite) than nuclear DNA. Cytochrome b gene in mitochondrial genome is utilized in many studies and not only in human *Plasmodium* (Steenkeste et al., 2009), but recently proven successful in microscopy negative samples in a newly rediscovered *Plasmodium* species of ungulates (Templeton et al., 2016). More PCR-based approaches including wide variety of target DNA or RNA transcripts and their limitations can be seen in recent reviews including Refs (Okell et al., 2012a, Bousema et al., 2014, Zimmerman and Howes, 2015).

### 1.13.4 Serology

There are several techniques for detecting anti-malaria antibodies in the serum. For each of the four *Plasmodium* species there are specific serological markers. A positive serological test is generally the indication of past infection. However, serology is not useful for detecting acute infection as detectable level of antibodies does not develop before week of infection and it persist long after the parasitaemia is cleared. In addition, serology is comparatively expensive and not widely available (WHO, 2011a).
1.14 Risk factors for malaria

The epidemiology of malaria is very complex, involving factors pertaining to the malaria parasites, the insect vectors, the human hosts, and the environment (van der Hoek et al., 1998). An understanding of the link between malaria transmission, climatic variables, and other human related factors is therefore necessary for developing appropriate measures that will significantly reduce transmission and perhaps eliminate malaria in endemic areas. In most cases these human related risk factors are known to aggravate the extent of climate related problems. Small variations in human related or environmental factors can have intense consequences for malaria transmission due to the variable status of immune status of the human population (Lindsay and Martens, 1998, Shankar, 2000). Different factors can drive these changes by influencing the vector’s transmission capacity and the malaria prevalence.

Malaria risk factors can be grouped into three classes:

1.14.1 Environmental factors

Environmental factors such as the presence of stagnant water around homes, presence of bushes, low altitude rainfall and high temperatures facilitate the breeding of malaria vectors, as well as parasite reproduction within vector (Messina et al., 2011), while increased urbanization tends to reduce the rate of Anopheles breeding.

Climate and environmental conditions greatly affect the transmission and incidence of malaria, by influencing primarily the abundance and survival of vectors and parasites, and exposure of humans and other hosts (Lafferty, 2009, Snow et al., 1999).

1.14.1.1 Temperature

Temperature effects the several stages of malaria life cycle. Many studies reported that optimal larval development (at 25°C-30°C), longevity and feeding frequency of
mosquito and stable malaria transmission are all temperature sensitive. The larval mortality increases when water temperature falls below 20°C (Bayoh and Lindsay, 2004). Speed of Plasmodium development within mosquito is also temperature dependent. The developmental cycle of malaria parasite within mosquitoes normally lasts for 9-10 days. The minimum temperature for mosquito’s development is between 8-10ºC, the minimum temperatures for parasite development are between 14- 19ºC. The optimum temperature for mosquitoes development is 25-27ºC, and the maximum temperature for both vectors and parasites development is 40ºC (McMichael et al., 1998). There are some areas with optimal climate for malaria parasite and mosquitoes but found malaria free. This can be attributed to the elimination of the Plasmodium or feeding habit of mosquitoes which does not feed on human blood. Any change in such areas that introduced the vector for human malaria can lead to destructive malaria outbreak due to lack of immunity in that population (Reid, 2000). In addition, higher temperatures increase the number of blood meals taken and the number of times mosquitoes lay eggs (Martens et al., 1995). An epidemic outbreak in Kenya was reported due to increased temperature (Kigotho, 1997).

1.14.1.2 Rainfall

Specific amount of precipitation is required for successful reproduction of Anopheles mosquito. Heavy rainfall or rainfall with storm condition can flush the breeding larvae (Paaijmans et al., 2007). Along with amount and intensity the time of rainfall in the year also affect the parasite survival. Malaria transmission is also affected by rainfall due to increase in relative humidity and change in temperature as it suggests where and in what quantities mosquito breeding can occur. The increase in humidity may be leading to an increase in adult mosquitoes longevity and consequently the vectorial
capacity (Warrell, 2002). A study conducted in highlands of Kenya reported the epidemics of malaria due to increased rainfall (Kigotho, 1997).

1.14.1.3 Altitude

A study to rank malaria risk factors in African highlands, suggested 1800–2000 meters as the upper limit at which malaria transmission could occur (Protopopoff et al., 2009, Warrell, 2002). Previously the upper limit of malaria in Pakistan was suggested to be 1,500 meters but recent malaria limit maps have shown malaria endemicity in areas between 1500 and 2000 meters (Baluchistan and KP) (MAP, 2013).

1.14.1.4 Topography

The distribution of hills, valleys, plateaus, rivers, streams, swamps, vegetation and poor drainage affects the spatial distribution of breeding sites (Minakawa et al., 2005), irrigation and swamp drainage for cultivation, can create new habitats for malaria vectors (Protopopoff et al., 2009). One of the reasons for the epidemic of malaria in an Afghan refugee camp in Khyber Pakhtunkhwa (KP), Pakistan was the borrow pits that were dugout for taking mud for building households, which may have been extra habitats for larvae breeding (Leslie et al., 2009a). In the Kenyan highland, most of the breeding habitats of the malaria vector in the hilly highlands were observed in the bottom of the valley, as the hillside gradients offer efficient drainage (Minakawa et al., 2004).

1.14.1.5 Wind

Wind may have both negative and positive impact on malaria cycle as very strong winds can decrease biting or ovipositing by *Anopheles*, while at the same time it can extend the flight length of the mosquito. During monsoon, wind can change the geographic distribution of mosquitoes (Reid, 2000).
1.14.1.6 Relative humidity

Relative humidity can disturb the malaria transmission by affecting the activity and survival of the *Anopheline* mosquitoes. If the average monthly relative humidity is below 60%, it is believed that the life of the mosquito is so shortened that malaria transmission is almost impossible. An epidemic outbreak in highlands of Kenya due to increased humidity was reported previously (Kigotho, 1997). Deforestation in Uttar Pradesh lead to reduction of humidity which results in short lifespan of *Anopheles* and clearance of *Plasmodium malariae* from that area as it requires long lifespan of vector host for its developmental period (Sharma, 1996).

1.14.2 Vector related factors

1.14.2.1 Vector Biology

High sporozoites rate and number of infectious bites are greatly influenced by the lifespan of the mosquitoes and mosquito density, which may lead to high transmission intensity (Paul et al., 2004, Protopopoff et al., 2009). The spread and survival of the malaria vectors are highly dependent on environmental factors (Adimi et al., 2010). Other important factors which can affect transmission of malaria in an area include; vector species e.g., zoophilic mosquitos which have preference for biting animals over humans (Bruce-Chwatt, 1960) (in Pakistan most of malaria vector species are zoophilic), climatic suitability for vector breeding and survival (Craig and Sharp, 1997). Number of different vectors, vector density, biting activity and entomologic inoculation rates (EIRs) are also the contributing factors for malaria transmission (Ndenga et al., 2006, Dev et al., 2004). Changes in vector biting behavior (Mboera and Kitua, 2001) and development of insecticide resistance in vectors (DMC, 2017) is also reported with increased risk of malaria.
1.14.3 Human related factors

1.14.3.1 Population immunity (ability to suppress infections)

Immunity is dependent on exposure to infection (Bodker et al., 2006), health condition of the individual and malnutrition (Shankar, 2000). Similarly, pregnant women are more susceptible to malaria. *Plasmodium falciparum*, which can sequester in the placenta causes severe morbidity to pregnant women and contributing significantly to maternal and infant mortality (Menendez, 1995). The other more susceptible group is HIV positive individual which are immune-compromised group and they might increase malaria transmission and malaria parasite biomass (Protopopoff et al., 2009). Previous study also reported that malaria incidence is quite low at first year of life due to the presence of maternal antibodies which provide partial immunity. After first year, the malaria incidence increase sharply and reached to maximum at 2-3 years of age. At this stage immunity seems to develop against both *Plasmodium* species but appears to mainly protective against *P. vivax* relapses (Stefani et al., 2011).

1.14.3.2 Personal Vector control

The personal protection (use of bed nets, mosquito repellent, and avoidance of mosquitoes during the peak biting times of the *Anopheles* mosquito) from vector bite can be very effective if followed on daily basis (Martens and Hall, 2000). However, the degree of efficacy differs individual wise depend on wide range of other factors.

1.14.3.3 Population migration

Huge population movement leads to population growth which results into socio-economic pressure, reduced access to health facilities and poor quality of health services (Martens and Hall, 2000, Hay et al., 2004) and all these circumstances are favorable for malaria outbreaks. Previous studies in Thailand and in the Philippines, had suggested periodic population movements into potential transmission sites in
endemic areas was the most important risk factor of increasing transmission (Fungladda et al., 1987, Sornmani et al., 1983). Population movement is also associated with the spread of drug resistance *Plasmodium* strains to areas previously free of drug resistance (WHO, 1999, Bloland, 2001).

**1.14.3.4 Antimalarial drug resistance**

Development of drug resistance is significant barrier in malaria control and it play an important role in spread of malaria to the new areas and in the occurrence and severity of epidemics (WHO, 2011a, Bloland, 2001). One of doubtless reason for resurgence of malaria in several countries was the appearance and spread of drug resistant strains (Bodker et al., 2000, Trape, 2001). Factors involved in emergence of drug resistance are human behavior, vector and parasite biology, pharmacokinetics and economics. In south Asian countries, the self-medication practice (Oemijati, 1992, Arasu, 1992, Fungladda and Sornmani, 1986) availing variety of health care practices and improper dosage intake have been suggested causes of drug resistance development (Fungladda and Sornmani, 1986). Drugs resistance leads to economic burden as resources are invested in development of new drugs (Foster, 1991, Ridley, 1997).

*Plasmodium vivax* and *Plasmodium falciparum* were reported with drug resistance to almost all existing antimalarial drugs while geographical distribution of resistance to any single drug varies greatly. *P. vivax* was found to acquire resistance to Chloroquine and/or primaquine in some areas (Murphy et al., 1993, Looareesuwan et al., 1997).

**1.14.3.5 Supra-Individual Level Risk Factors**

Malaria transmission is also influenced by culture, regional and household economics, geographic location, and community support mechanism (Diez-Roux, 1998, Mauny et al., 2004). These supra-individual level factors can provide the background for malaria
existence in a population and help in understanding that why malaria affect the individuals differently.

The distance between houses and vector breeding sites (Staedke et al., 2003, Vittor et al., 2006, Tilaye and Deressa, 2007, Graves et al., 2009, Vittor et al., 2009), socioeconomic status of individuals (Somi et al., 2007, Gamage-Mendis et al., 1991), regional conflicts (Messina et al., 2011), number of family members, houses building material (Ghebreyesus et al., 2000, Butraporn et al., 1986, Arasu, 1992, Sornmani, 1992), literacy level of family members or head of the family and presence of domestic animals inside house or near the house (Bogh et al., 2001) and human travel (Siri et al., 2010) are few examples of supra-individual factors. Community level risk factors included access to health facilities (Kreuels et al., 2008, Tanner and Vlassoff, 1998) proportion of individuals over 45 years age, community-wide fumigation campaigns (Peterson et al., 2009). Along with individual level factor the supra-individual level factors also provide significant information about malaria transmission as it is reported that social context can greatly influence the ones’ health and his health outcomes (Duncan et al., 1993).

## 1.15 Genetics of *Plasmodium*

Genetic structure of any parasite is useful for understanding of evolution of parasite virulence, change in parasitic characteristics, development of vaccines and assessing the impact of malaria control measures (Thakur et al., 2008, Mzilahowa et al., 2007). Unfortunately, there is lack of knowledge about genetic structure of existing *Plasmodium* species (*P. vivax* and *P. falciparum*) in Pakistan (Khatoon et al., 2010). The study of genetic diversity can provide significant evidence on a parasite’s response to human interventions for example drugs or vaccines, as directional selection will favor the fixation of advantageous alleles in population thus reducing genetic diversity (Paul
et al., 1995). A very limited information is available on genetic structure of *Plasmodium vivax* and *Plasmodium falciparum*. One recent study analyzes the genetic diversity of *Plasmodium vivax* in FATA region of Pakistan (Zakeri et al., 2010d).

Among human malarial parasites, *P. vivax* causes maximum illness outside Africa (WHO, 2014a). *P. vivax* differs from the more widely studied *P. falciparum* in aspects of its geographic distribution, evolutionary history, life cycle, disease severity, ecology and raising concerns that gaps in our knowledge about its basic biology may compromise its control (Galinski et al., 2013, Prajapati et al., 2013, Schneider and Escalante, 2013, Otten et al., 2009, Escalante et al., 2005, Prugnolle et al., 2013, Mueller et al., 2009). The control and ultimate eradication of *P. vivax* are global health priorities. Genomic research contributes to this objective by improving our understanding of the biology of *P. vivax* and through the development of new genetic markers that can be used to monitor efforts to reduce malaria transmission (Winter et al., 2015).

Arrival of drug resistant strains indicates the genetic mutation of parasite. Drug resistance results into increased virulence of the *Plasmodium vivax* which warns the attention of medical practitioners to both species. The genetic variations play an important role in parasite’s survival strategies including the potential for recombination, clonal expansion, gametocyte production drug resistance and escape from host immune response (Nassir et al., 2005, Lee et al., 2006). The best way of finding genetic diversity is to analyse more than one marker gene, in such manner the probability that different clones share the same genotype by chance is considerably reduced (Schoepflin et al., 2009).

Due to high mortality rate and morbidity, the *Plasmodium falciparum* put the extreme selective pressure on human genome in recent past. Several genetic factors were
reported to show resistance to *Plasmodium* infection such as sick cell traits, absence of Duffy antigens on red blood cells and Glucose-6-phosphae dehydrogenase deficiency (Kwiatkowski, 2005, Hedrick, 2011).

The migration of *Plasmodium* parasite from one geographical region to other have become very frequent due to increased population movement (within the country, cross country and cross-continent). Such migratory phenomena of human malaria pathogen is generally associated with disease epidemics (Engering et al., 2013) and addition of new and recombined genotypes in the local population. These genetically recombined parasite genomes often give rise to new virulent and drug-resistant phenotypes (Darch et al., 2015).

In case of sudden malaria outbreak or unexpected emergence of anti-malarial drug resistance to a working drug, the identification of parasite genotype is of utmost importance in controlling the drug resistance and outbreaks. The exact geographical origin and migration route of pathogen should be exactly known for effective intervention and control measures. For successful treatment, it is necessary to know whether the patient is parasitized with single or multiple clones of parasite, as in high transmission region or season the chances of multiple clonal infection is quite high. Accurate characterization of *Plasmodium* species is also very important to monitor the frequency and distribution of specific species in population and differentiate recurrent infection from re-infection in drug trials (Tripathi and Das, 2015).

In such circumstances the genotyping of parasite is considered the only way to distinguish between various clones. Initially parasite clones were differentiated by nested Polymerase chain reaction (PCR)(Snounou et al., 1993b). On the basis of length polymorphisms at two merozoite surface protein genes (msp-1 and msp-2), one gene encodes for glutamine-rich protein (glurp) and other is circumsporozoite protein-
encoding gene (csp). Recently SNPs (located in the conserved regions of nuclear or extra-nuclear genomes) approach provide a new way for future genotyping of malarial parasites (Tripathi and Das, 2015).

Genomic approaches also make easier the study of parasites which are hard to culture such as *Plasmodium vivax*. For example, genome-wide scans performed on samples from a natural parasite population can identify regions of the genome subject to strong selection (Mu et al., 2010, Ochola et al., 2010, Amambua-Ngwa et al., 2012, Park et al., 2012). Previous studies of population genetic have reported that *P. vivax* populations in many regions of the Americas are less diverse than those from Asia or Oceania (Jongwutiwes et al., 2005, Cornejo and Escalante, 2006, Imwong et al., 2007, Chenet et al., 2008, Cullerton et al., 2011). Similarly, a current whole genome study documented limited genetic diversity in a population of Amazon basin of Peru (Flannery et al., 2015). Genomic studies can also support efforts to control and eliminate malaria from a given region by identifying genetic markers that will be informative for fine-scale population genetic studies in that region. Molecular epidemiological investigations rely on multilocus genotyping of SNPs or microsatellites (Escalante et al., 2015) to investigate patterns of population structure and gene flow.

Whole genome study helps in identification of new polymorphic loci which helps in the pattern of transmission and can be used to distinguish the local cases from those introduced from other regions. Limited sequence analyze of protein coding regions of nuclear origin have revealed the difference between local and imported pathogens as well as between parasite strains imported from different endemic regions (Severini et al., 2002).
As a source of information on *P. vivax* polymorphisms, *Plasmodium* mitochondrial genome (6-kb) is comparatively more useful than currently available nuclear markers for epidemiological studies. For epidemiological studies the mitochondrial genome of the *P. vivax* is more useful than currently available marker as a source of information on polymorphism due to some advantages such as; a). The mitochondrial genome does not recombine with each other due to uni-parental inheritance, b). Mitochondrial haplotypes can be clustered per their geographic origin, c). Most of the polymorphisms in mitochondrial genome are evolutionary neutral and they reflect the population history of lineage more effectively than polymorphism in coding region (Conway et al., 2000, Joy et al., 2003).

Mitochondrial genome sequencing was proved to be useful at population level studies in many organisms specifically in case of *Plasmodium* genus. Mitochondrial genome consists of many genes encoding for ribosomal RNA and three genes for cellular respiration i.e. Cox I (cytochrome c oxidase gene I), Cox III (cytochrome c oxidase gene III) and Cytb (cytochrome b). The later three genes are crucial for cellular activities such as oxidative phosphorylation, heme and coenzyme Q biosynthesis and maintenance of membrane potential (Hughes, 2004).
1.16 Rationale of the project:

The aim of this study is to generate current information on malaria infection through prevalence of malaria infection among the suspected individuals of District Bannu (where transmission of malaria is perennial) in Khyber Pakhtunkhwa (KP) province of Pakistan formerly called North West Frontier Province (NWFP).

*P. falciparum* and *P. vivax* malaria are the major health issue in Pakistan. At least 39 districts of the two southern provinces of Balochistan and Sindh have been classified as high risk area. In different areas of Pakistan studies have been conducted on prevalence of *Plasmodium* (Khatoon et al., 2009, Yasinzai and Kakarsulemankhel, 2004, Niazmani et al., 1995) but few studies were conducted in the study area (Bannu) related to malaria prevalence. To fulfill this gap the present study was conducted to assess the epidemiology of malaria in the region. The disease is endemic in different parts of Khyber Pakhtunkhwa, including the study site. The present study was designed with the main aim of investigating the prevalence of malaria infection among the suspected individuals of Bannu district, Khyber Pakhtunkhwa, Pakistan and creating awareness of malaria disease in them.

The epidemiology of malaria is very complex, involving factors pertaining to the malaria parasites, the insect vectors, the human hosts, and the environment (van der Hoek et al., 1998). An understanding of the link between malaria transmission, climatic variables, and other human related factors is therefore necessary for developing appropriate measures that will significantly reduce transmission and perhaps eliminate malaria in endemic areas. This study was also aimed to recognize the possible risk factors of malaria, its relationship with infection and exposure which will be helpful in the prevention and control of the disease in this area and will also assist decision makers in better targeting malaria intervention and control efforts. Identified risk factors and
malaria incidence patterns can be utilized in devising localized disease control initiatives.

Additionally, the clinical isolates obtained from District Bannu, Khyber Pakhtunkhwa were typed for mitochondrial genome to elucidate the status of *P. vivax* epidemiology in south region of Khyber Pakhtunkhwa province of Pakistan in relation to the global samples.

In case of sudden malaria outbreak or unexpected emergence of anti-malarial drug resistance to a working drug, the identification of parasite genotype is of utmost importance in controlling the drug resistance and outbreaks. The exact geographical origin and migration route of pathogen should be exactly known for effective intervention and control measures. For successful treatment, it is necessary to know whether the patient is parasitized with single or multiple clones of parasite, as in high transmission region or season the chances of multiple clonal infection is quite high. Accurate characterization of *Plasmodium* species is also very important to monitor the frequency and distribution of specific species in population and differentiate recurrent infection from re-infection in drug trial (Tripathi and Das, 2015). Simple genetic markers from apicoplast and mitochondrial genomes can be utilized in studying genetic profiles or haplotypes from *P. vivax* that can be specific to their geographic origin and thus can be a valuable public health tool for locating the source of outbreaks. Such haplotypes for *P. vivax* can have prospects in monitoring drug resistance and epidemiology of these specific profiles in a region and identification of specific genotypes will help to map out import routes and thus implement proper disease general control measures by the Health department.
Specific Objectives:

The major goals of this research are to:

1. Estimate incidence of malaria in Bannu district, using three diagnostic methods i.e. Microscopy, RDT, and PCR.
2. Evaluate the specificity and sensitivity of the employed diagnostic means in the study area.
3. Assess behavioral and household risk factors for malaria in Bannu, district.
4. Genotype and sequence apicoplast and mitochondria geographical-specific markers in *P. vivax*.
Chapter 2  MATERIALS AND METHODS

2.1  Introduction

Bannu (32°43′–33°06′ N; 70°22′–57′ E) is one of the 26 districts in the south of Khyber Pakhtunkhwa province of Pakistan with an area of about 1227 sq. km and a population of 1,167,892 (Pakistan Bureau of Statistics, 2017) majority of which is rural. Bannu is divided into two tehsils Domel/Bannu-1 and Bannu-II, comprising 40 union councils (Government of Khyber Pukhtunkhwa, 2017). About 80 public health centers including Medical Teaching Institutes (MTI), District Headquarter Hospitals (DHQ), Regional Health Centers (RHC), Basic Health Units (BHU), Civil Dispensaries (CDs) and Mother & Child Health Centers (MCH) along with 15 private centers operate in Bannu District. In these health centers, first line treatment for unconfirmed malaria is Chloroquine, for *P. vivax* is Chloroquine-Primaquine, for uncomplicated *P. falciparum* malaria is Artesunate/ Sulfadoxine-Pyrimethamine (AS+SP), whereas Artesunate, Artemether or quinine is recommended for treating severe *P. falciparum* malaria or cases with treatment failures (WHO, 2012). Control interventions being conducted here are run and sponsored by “Integrated vector control/ malaria control program Khyber Pakhtunkhwa” (IVC/MCP-KP) and “Frontier Primary Health Care” (FPHC). These include regular trainings for focal persons in basic microscopy, RDTs use, malaria case management training, outbreak training etc. Other chief prevention strategies include indoor residual spray, mass distribution of bed nets to every household, antenatal care (ANC) bed net distribution (only pregnant women receive a bed net during her visit to hospital for antenatal care) and community education and mobilization campaigns (*Personal communication* with IVC/MCP-KP).
Table 2.1 Demographic information of District Bannu

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<td><strong>Area</strong></td>
<td>1,227 km² (474 sq mi)</td>
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<tr>
<td><strong>Population - 2017</strong></td>
<td>1,167,892 persons</td>
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<tr>
<td>• Male</td>
<td>593,492</td>
</tr>
<tr>
<td>• Female</td>
<td>574,391</td>
</tr>
<tr>
<td><strong>Population Density</strong></td>
<td>950/km² (2,500/sq mi)</td>
</tr>
<tr>
<td>• Urban Population</td>
<td>499,655</td>
</tr>
<tr>
<td>• Rural Population</td>
<td>1,117,927</td>
</tr>
<tr>
<td><strong>Literacy Ratio (2017)</strong></td>
<td>66.02</td>
</tr>
<tr>
<td>• Male</td>
<td>50.79 %</td>
</tr>
<tr>
<td>• Female</td>
<td>12.18 %</td>
</tr>
<tr>
<td><strong>Population - 1981</strong></td>
<td>423,018 persons</td>
</tr>
<tr>
<td><strong>Total Housing Units</strong></td>
<td>119,993</td>
</tr>
</tbody>
</table>

**Administrative Units**

- Tehsils: 02
- Union Councils: 40
- Mauzas: 229
- Municipal Committees: 01
- Cantonment: 01

(Government of Khyber Pukhtunkhwa, 2017)

Figure 2.1 (a) Map of Pakistan showing its provincial divisions and neighboring countries (b) Map showing administrative divisions of KP and FATA

*Encircled region showed study area*
2.2 Ethical approval

Patients were dually informed about the purpose of sample collection and written consent was obtained for inclusion in the study. Results of the different malaria diagnostic tests were communicated to the patients and were further guided about any consequent recommended medication. The study procedures have been approved by Peshawar University (local Ethics committee) and copy of ethics clearance is attached in appendix A.

2.3 Data collection and sampling procedure

Blood samples were collected from suspected malaria patients (belonging to different parts of Bannu district) visiting Malaria Model Laboratory, Malaria Lab (Women & Children Hospital Bannu) and Siddique Laboratory Bannu for diagnosis or treatment purposes. The Labs catchment area covers the entire district. The flow of patients was greater in these labs as among above mentioned labs two (MML and Malaria Lab WCH) are govt. labs which provide free services with collaboration of UNHCR malaria control program. While the later one has good reputation for satisfactory diagnostic facility. Samples were collected from March to October 2013. Sample collection was performed thrice in a week. Patients, suspected of malaria, visiting the above-mentioned labs were taken in for sampling. Primary data was collected from the patients through a questionnaire for acquiring demographic and other relevant information including gender, literacy level, locality, socioeconomic status etc. for assessing risk factors for malaria in the study area (Appendix B). As questionnaire was in English, the scale was translated into Urdu & Pushto. For children, consent was requested from a parent or an adult guardian. Each patient (or guardian) was interviewed in person by principal investigator. Anonymization was maintained by omission of names of participants and their replacement with ID numbers.
Ethyl alcohol, glass slides, RDT kit, filter papers (for blood spot collection) and cooling box with ice packs were carried along in each visit to the laboratory. Filter papers were prepared in laminar flow hood (Department of Zoology, University of Peshawar) by cutting about 2cm pieces and each piece was stapled between card sheets.

Patient finger was sanitized with alcohol, allowed to dry and later pricked with blood lancet. Blood was processed with RDT (First response® Malaria Ag, pLDH/HRP2 Combo Card test kit, Cat. No 116FRC30), microscopy (thick and thin smears) (WHO, 2009) and PCR for the confirmation of *Plasmodium* species. Few drops of blood were placed on glass slide for making smears utilized for microscopy, while one drop was taken by disposable pipette and placed on RDT. To detect the malaria parasite using PCR about 1 micro liter of blood was spotted (2-3 spots) on coarse-porosity filter papers. These filter papers were stored in small zip lock bags with silica beads at low temperature (4°C). The serial number assigned to the subject and collection date was written on a card cover placed along the filter paper. These samples were transported to the molecular lab of Department of Zoology; University of Peshawar. These filter papers were utilized in identifying *Plasmodium* species using PCR.

Results of the different malaria diagnostic tests was communicated to the patients and they were further guided about any consequent recommended medication.

### 2.3.1 Microscopy

Malaria parasite is found in the red blood cells of human beings. The diagnosis of malaria is dependent upon the presence of malaria parasite in the peripheral blood film. The essential requirements for a precise diagnosis of a case of malaria are: Making a proper blood smear from a suspected malaria patient, high quality staining of the smear and examination of the stained slide by a skilled microscopist.
Two types of smears were prepared from the peripheral blood i.e. thin and thick smear. Thick film examination is sensitive for the presence of parasite. Thin film examination was made to identify the species of *Plasmodium*. For preparation of blood smear the patient finger was cleaned with alcohol and blood drops (3 for thick smear and 1 for thin smear) were taken on slide, 1 cm apart from each other. With the help of another slide (spreader) thick smear was made by joining the 3 drops of blood and spreading over an area of 10 mm diameter. Thin smear was made by bringing the spreader in contact with the drop of blood at an angle of 30-45º from the horizontal and pushing the spreader gradually down the surface of the slide drawing the blood behind till the smear was formed. Slides were number after drying and later fixed with methanol. All slides were fixed with Giemsa stain (mixture of eosin and methylene blue) by placing in staining trough for 30-45 minutes. Stain was poured off gently and rinse the slides with clean water for a few seconds and then allowed for drying. Thick film was examined first and thin film was examined only when the thick film gets auto fixed or when it is necessary to confirm the identification of a species. The examination continued, for at least 100 fields to determine whether the blood film is positive or negative for malaria (WHO, 2009).

**Figure 2.2 Thick and thin smear**
2.3.2 Rapid Diagnostic Technique (RDT)

Rapid tests were conducted by using rapid diagnosis kits. (First response® Malaria Ag, pLDH/HRP2 Combo Card test kit, Cat. No 116FRC30). The CSPfPan test was suggested the most appropriate for this P. vivax predominant region and was found to be the most sensitive of the RDTs compared which was not unduly affected by heat (Mikhail et al., 2011). 5ul fresh blood was taken through company provided pipette and released into sample well and put two drops of buffer solution (amount required per type of RDT). The RDT strip was left for 20 minutes to progress as suggested by the manufacturers. After 20 minutes’ results were read and interpreted according to RDT results interpretation chart. All positive cases were further confirmed by microscopy.

2.3.3 Sample storage

3-4 spots were taken on filter paper and left for few minutes to become air dry then labelled with patient ID and date of collection. Disposable gloves were used during sample collection. At the end of the day the filter papers were wrapped individually in an airtight re-sealable bag with silica gel. These filter papers were carried in cold storage boxes and then stored in the fridge, later transferred to the -20°C at the Dept. of Zoology, University of Peshawar. All blood samples collected on filter paper were packed and transferred in ice box containing ice packs to department of zoology university of Peshawar and preserved in -20°C.

2.3.4 Molecular diagnosis

PCR was used to identify malaria infections. Filter paper samples including all microscopy positive and negative samples were processed by PCR (Snounou et al., 1993a).
DNA Extraction

A resin based method (employing Chelex) was used for DNA extraction described by Plowe, *et al.*, 1995. DNA was extracted from the blood samples on filter paper using the resin-based Chelex method as follows. Sections of the filter papers were punched using Harris Uni-Core hole punch (at least 2mm diameter). About 1-2 discs were collected in each separate autoclaved Eppendorf tube (marked by sample ID). The punch hole and the forceps were treated with 70% alcohol and flame between each sample. In each batch about 30-40 samples were processed. Spots were immersed in 1ml freshly prepared 0.5% Saponin in 1 x Phosphate buffered saline (PBS). All tubes were tightly closed and then incubated at 37°C overnight to release the haemoglobin in to the PBS, leaving the DNA on the paper. Each sample was briefly centrifuged (4000 rpm for 2 min). Saponin and debris were removed by micropipette with a new tip for each sample. 1ml fresh 1 x PBS was added to each tube and centrifuged (4000 rpm for 2 min) and washing step was repeated until no haemoglobin was seen on the filter papers. 150μl of 6% Chelex-100 suspension in nuclease-free water (using a trimmed pipette tip) was added. All samples were boiled for 30 minutes in a water bath, ensuring that the level of water covers the samples in the tubes. Each tube was spun at 4000 rpm for 2 minutes to pellet down the Chelex and the filter papers. The DNA supernatant (approximately 120μl of the top layer) was transferred into sterile pre-labelled tubes. Each tube was spun down again (4000rm for 10mins) to sediment any Chelex taken in with the DNA. Labelled Eppendorf tubes containing DNA were stored at -20°C and Chelex and filter paper was discarded. All samples of one batch were stored in one pack labelled with Date, time, number of samples at -20°C.
Diagnostic PCR for identification of *Plasmodium* species

Nested PCR targeting conserved rRNA genes was utilized for *Plasmodium* species discrimination using genus and species specific primers as described by (Snounou et al., 1993a). A 130 bp fragment of the *Plasmodium* spp. 18S genes was amplified using 10 pmoles of each Genus-specific primer.

For first amplification cycle genus-specific primers rPLU5 and rPLU6 were used. The first amplification cycle product was then used for a second amplification cycle, in which each parasite species is identified separately using species-specific primers.

**Table 2.2 Primers used in PCR**

<table>
<thead>
<tr>
<th>Purpose</th>
<th>Primer</th>
<th>Forward primer (5’-3’)</th>
<th>Primer</th>
<th>Reverse primer (3’-5’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genus specific</td>
<td>PLU 6</td>
<td>TTAAAATTGTGGCAGTTA AAACG</td>
<td>PLU 5</td>
<td>CCTGTGTGTGCCTAAAC TTC</td>
</tr>
<tr>
<td>Species specific</td>
<td>rVIV2</td>
<td>ACTTCCAAGCCGAAGCAA AGAAAGTCCCTTA</td>
<td>rVIV1</td>
<td>CGCTTCTAGCTTAAATCCA CATAACTGATAC</td>
</tr>
<tr>
<td></td>
<td>Rfal2</td>
<td>ACACAATGAACCTCAATCA TGACTACCGTC</td>
<td>Rfal1</td>
<td>TAAAACGTGGTTGGGAA AACCAAATATATT</td>
</tr>
</tbody>
</table>

**Nest 1 Reaction**

The PCR was nested using sets of primers as described by Snounou et al. (Snounou et al., 1993a). The Nest 1 PCR mix and cycling conditions were as follows
Table 2.3 PCR Mix for Nest 2 reaction

<table>
<thead>
<tr>
<th>Working concentration</th>
<th>Final concentration</th>
<th>Reaction volume 20 µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH2O</td>
<td>-</td>
<td>10.5 µl</td>
</tr>
<tr>
<td>10XNH₄SO₄ buffer</td>
<td>1X</td>
<td>2 µl</td>
</tr>
<tr>
<td>50 mM MgCl₂</td>
<td>2 mM</td>
<td>0.8 µl</td>
</tr>
<tr>
<td>10 mM dNTPs</td>
<td>250 µM</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>5 µM Primer mix (genus specific)</td>
<td>250 nM</td>
<td>1 µl each</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td></td>
<td>0.2 µl (1 unit)</td>
</tr>
<tr>
<td>DNA</td>
<td>-</td>
<td>5 µl</td>
</tr>
</tbody>
</table>

The cycling parameters for the first amplification reaction were as follows:

The cycling conditions for N1 are as follows,

Table 2.4 cycling conditions for Nest 1

<table>
<thead>
<tr>
<th>Step</th>
<th>Temp (°C)</th>
<th>Time (min.)</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step-1</td>
<td>95</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Step-2 Annealing</td>
<td>58</td>
<td>2</td>
<td>25 cycles</td>
</tr>
<tr>
<td>Step-3 Extension</td>
<td>72</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Step-4 Denaturation</td>
<td>94</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Step-2 Annealing</td>
<td>58</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Step-3 Extension</td>
<td>72</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>10</td>
<td>1</td>
</tr>
</tbody>
</table>

Nest 2 Reaction

PCR mix and cycling conditions for N2 are as follows.

**PCR Mix** (PCR mix for N2 was prepared according to the method as mentioned above).
Table 2.5 PCR mix for Nest 2

<table>
<thead>
<tr>
<th>Working concentration</th>
<th>Final concentration</th>
<th>Quantity per 20 µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH₂O</td>
<td>-</td>
<td>14.5 µl</td>
</tr>
<tr>
<td>10XNH₄SO₄ buffer</td>
<td>1X</td>
<td>2 µl</td>
</tr>
<tr>
<td>50 mM MgCl₂</td>
<td>2 mM</td>
<td>0.8 µl</td>
</tr>
<tr>
<td>10 mM dNTPs</td>
<td>250 µM</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>5 µM Primer mix (P. falciprum or P. vivax specific)</td>
<td>250 nM</td>
<td>1 µl</td>
</tr>
<tr>
<td>Taq</td>
<td>1 unit</td>
<td>0.2 µl</td>
</tr>
<tr>
<td>DNA (N1 product)</td>
<td>-</td>
<td>1 µl</td>
</tr>
</tbody>
</table>

Cycling conditions: The cycling parameters for the second amplification reaction were as follows:

Table 2.6 cycling conditions for Nest 2

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temp (°C)</th>
<th>Time (min.)</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step-1</td>
<td>95</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Step-2 Annealing</td>
<td>58</td>
<td>1</td>
<td>30 cycles</td>
</tr>
<tr>
<td>Step-3 Extension</td>
<td>72</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Step-4 Denaturation</td>
<td>94</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Step-2 Annealing</td>
<td>58</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Step-3 Extension</td>
<td>72</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>10</td>
<td>1</td>
</tr>
</tbody>
</table>

For preparation of 2% agarose gel, 2 mg of agarose was dissolved in 100ml of 1x Tris-Borate-EDTA (TBE). Heated the mixture in microwave to dissolve agarose particles then added 2μl of Ethidium Bromide (10mg/ml) and mixed it. Then boiled the mixture until transparent solution was obtained. Cooled up to 50°C before pouring into gel tray. Allowed the gel to set for 30 minutes at room temperature and then combs were removed. N2 samples (10μl), each were added into respective vials along with loading dye (2μl). 6μl ladder (100bp) was added into 1st and another selected veil. Power supply was set at 90 volts for 60 minutes. After separation of nest 2 products, gel was visualized.
in gel doc under UV light to see the *Plasmodium* species bands. *Plasmodium falciparum* produces 205 bp PCR product and for *Plasmodium vivax* the PCR product size produced is 120bp.

### 2.4 Sequencing of mitochondrial genome

Mitochondrial genome was sequenced for *Plasmodium vivax*. For this purpose, a set of DNA samples identified as *P. vivax* from the present study (N=60). These samples were sent to London School of Hygiene and Tropical Medicine (LSHTM) for sequencing where 11 novel primers were utilized for sequencing of mitochondrial genomes (approx. 6Kb) of the Pakistani *P. vivax* samples (table 2.7).

#### Table 2.7. List of primers used for mitochondrial genome sequencing

<table>
<thead>
<tr>
<th>No.</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1F:AGCTGTAAGATGGATGCTTCGA</td>
<td>1R:ACCCTAGAACATTAGAACAAGGAG</td>
</tr>
<tr>
<td>2</td>
<td>2F:TGGCATCTCTTCGATTTCCA</td>
<td>2R:ACAGGCTGATGCTCTATGCCC</td>
</tr>
<tr>
<td>3</td>
<td>3F:TTCCCCGGTGATCCAATCCAG</td>
<td>3R:AATGGCGGAGAAGGAAGTGT</td>
</tr>
<tr>
<td>4</td>
<td>4F:GACCAGATCAAACGGGAATCA</td>
<td>4R:TTGCACTAATTCAACAAACTGA</td>
</tr>
<tr>
<td>5</td>
<td>5F:GAACGGTGATTTTGIGTGC</td>
<td>5R:GATCCATACAGTCCCAGGCA</td>
</tr>
<tr>
<td>6</td>
<td>6F:GCTGGGACTGTATGGATCGA</td>
<td>6R:TCTCCAGCAAAATGATGATCAA</td>
</tr>
<tr>
<td>7</td>
<td>7F:TGTCTTTATCTCTGTCAG</td>
<td>7R:TTGTCTTTGTATCTACTTCAACCTG</td>
</tr>
<tr>
<td>8</td>
<td>8F:AGGAAGTTGTATGGGCTCA</td>
<td>8R:CCATCCATTTAAAGCGTCTGGA</td>
</tr>
<tr>
<td>9</td>
<td>9F:TGTAAATTCTAGCTAGTATTCGTA</td>
<td>9R:CCCAATAACTCTATTGTCCTCC</td>
</tr>
<tr>
<td>10</td>
<td>10F:GGGGACAAATGATGTTGAGCGT</td>
<td>10R:CGGTCTGTATTGTTCTGCTCA</td>
</tr>
<tr>
<td>11</td>
<td>11F:ATGGATTTGGATGTCAGCTACA</td>
<td>11R:GTTTAGCCAGGAAGTGCAGCG</td>
</tr>
</tbody>
</table>

### 2.5 Data analysis

Questionnaire data was entered into Microsoft excel and were analyzed in IBM SPSS Statistics 20. The data was analyzed with Stata software, version 14. Cross tabulation and different tests for comparison of diagnostic techniques including Cohen’s kappa, McNemar marginal homogeneity test were carried out in IBM SPSS Statistics 20.
2.5.1 Assessment of sensitivity and specificity of diagnostic methods

Agreement of results between the two methods was assessed using Cohen’s k-test (Cohen 1960) for concordance and McNamara’s test for discordance. Kappa statistic is frequently used to test interrater reliability. It represents the extent to which the data collected in the study are correct representations of the variables measured. Cohen’s kappa, symbolized by the lower case Greek letter, κ (Marston, 2010) is a robust statistic useful for either interrater or intra-rater reliability testing. Like correlation coefficients, it can range from −1 to +1, where 0 represents the amount of agreement that can be expected from random chance, and 1 represents perfect agreement between the raters. While kappa values below 0 are possible, Cohen notes they are unlikely in practice (Marston, 2010). Kappa result be interpreted as follows: Values ≤ 0 as indicating no agreement, 0.01–0.20 as none to slight, 0.21–0.40 as fair, 0.41–0.60 as moderate, 0.61–0.80 as substantial and 0.81–1.00 as almost perfect agreement (Landis and Koch, 1977).

McNemar's test is a statistical test used on paired nominal data. It is applied to 2 × 2 contingency tables with a dichotomous trait, with matched pairs of subjects, to determine whether the row and column marginal frequencies are equal (that is, whether there is "marginal homogeneity"). It is named after Quinn McNemar, who introduced it in 1947 (McNemar, 1947).

For the different diagnostic methods used in the study sensitivity (Probability of a patient being test-positive when disease present i.e. sensitivity=true positive/true positive + false negative), specificity (Probability of being test-negative when disease absent i.e. Specificity = true negative/true negative + false positive), positive predictive value (PPV) (Probability of patient having disease when test is positive i.e. PPV=true positive/true positive + false positive) and negative predictive value (NPV) (Probability
of patient not having disease when test is negative i.e. NPV=true negative/false negative + true negative) were calculated. Sensitivity and specificity are inversely proportional, meaning that as the sensitivity increases, the specificity decreases and vice versa (Parikh et al., 2008). All samples were considered confirmed positives if they were positive parasitologically by at least two diagnostic methods. Samples were considered confirmed negatives if they were negative by one method.

2.5.2 Multivariate and univariate analysis for risk factors

Among Two thousand and thirty-three individuals, positive cases by all diagnostic means were matched by gender and age categories (<5, 5-20, >20 year) to derive 359 matched pairs in Stata version 13 (Statacorp, 2013). Using this paired data, risk factor assessment was performed through univariable and step-wise multivariable conditional logistic regression in Stata v13.

Univariate and multivariate logistic regression model was used, to investigate whether socio-economic and demographic factors as a risk factors of malaria prevalence of infection and exposure in the studies population. Potential risk factors tested in the models were: age in years, sex, literacy level, malaria fever history, intake of antimalarial drugs, previous malaria history, present symptoms, water reservoir near to house, house construction material, window glazing, recent or regular bed net use, and use of repellents or coils and socio-economic status (SES) as an indicator of household wealth. To determine the effect of each exposure variable on the outcome variable univariate analysis was performed. Any exposure variable significant with p≤ 0.05 in univariate analysis was used in multivariate analysis (Wald test). The final multivariate modal includes only significant exposure variable with p≤ 0.05.
2.6 Analysis of mitochondrial genome data

Mitochondrial sequences from 11 primers generated from *P. vivax* samples were merged and aligned against complete genome of *P. vivax* reference strain Salvador I. Missing data between and within sequences were represented N. All sequences were aligned in MEGA v7 using Clustal W alignment and all ambiguous site with insertion or deletion were removed. All SNPs were identified and made compatible with secondary SNP data of 210 *Plasmodium* isolates from 17 countries (kindly provided by Dr. Taane Clark, LSHTM) that worked as backdrops for genetic analysis of Pakistani samples.

Composite mitochondrial sequences from Pakistan were translated in GENEIOUS v10.2.3 to identify as synonymous and non-synonymous mutations in mitochondrial genes (Cytochrome B, COX-1 and COX3). Translation was done with genetic code of translation table-4 of Mold protozoan mitochondrial genome. By comparing our sample sequences with reference sequence of Salvador I, SNPs were exported to Excel sheet along with those other countries.

2.6.1 Identification of genetic population substructure

Identification of the number of clusters within the SNP dataset was approached by 2 methods i.e. STRUCTURE and NJ tree (Neighbor Joining tree).

2.6.1.1 STRUCTURE approach

STRUCTURE V2.3.1 was used. The program structure uses a Bayesian model-based clustering method for inferring population structure using MCMC (Markov chain Monte Carlo) scheme (Pritchard et al., 2000). The model assumes that within the populations the marker loci are unlinked and at linkage equilibrium with one another. It also assumes the populations are in Hardy-Weinberg equilibrium (HWE). It is thus
not considered appropriate to be used for diploid organisms although, the versions 2.0 onwards have been modified to some extent to deal with weakly linked markers (Pritchard et al., 2009).

The model also does not assume a mutation process and thus can be applied for commonly used markers as microsatellites, RFLPs or SNPs etc. The method can be used to identify population structure and migration or admixture events. Isolates are assigned probabilistically to one population or more (in case of admixed genotypes) based on a set of allele frequencies characteristic for each population.

K (number of clusters) may be predefined or calculated by the software. To calculate K, the number of iterations were set to 100 000 iterations with additional 500 000 MCMC iterations. Admixture ancestry model was used to infer Alpha (α) set between 1.0-10.0 with a standard deviation of 0.025. Same value of alpha was used for all sub-populations. The settings for frequency model were (a) Allele frequencies correlated among sub-populations (b) Different values of FST for different sub-populations (c) Constant value of the parameter for allele frequencies (λ). (d) The frequency of Metropolis update for Q (estimated membership coefficient) as 10 runs for each value of K. The value of K was allowed to vary between 1-20 for 5 independent runs. The mean of LnP(D) (estimated log likelihood values ln Pr(K/X), where X is the number of genotypes) for each K was then plotted against the number of clusters (K). The value of optimum K, is chosen where the graph ceases to follow a Gaussian distribution so that K value increases and more-or-less plateaus at some point. If two or more values of K give almost similar log Pr(X|K) figures then the smallest of these is chosen as the optimum number of sub-populations (Pritchard et al., 2009).
2.6.1.2 Neighbor joining phylogenetic tree

The NJ tree building method is heuristic approach that uses genetic distances to infer the cladistic clustering of similar isolates. This algorithm assumes different rates of evolution for different lineages. However, if the evolutionary rate varies greatly among sites or if some distances are large then accurate estimation of the optimal tree becomes difficult. Otherwise the method is well known for its robustness (Page and Holmes, 1998b).

The fasta format file created from MEGA v5.05 was run in jMODELTEST v1.0 to select a best fit nucleotide substitution model from a list of 88 available (Posada, 2008). The best model selected for the dataset was used to construct a Maximum Likelihood tree (ML) tree in MEGA V5.05 with 1000 bootstrap re-samplings.

2.6.2 Population genetic analysis of geographical populations

The all available populations were divided into geographical clusters on the basis of WHO malaria regions (WHO, 2017). Several sub-population parameters were calculated. This analysis was carried out on geographically assigned clusters/sub-populations in table 2.7.

Table 2.7 Distribution of populations according to WHO malaria regions

<table>
<thead>
<tr>
<th>Cluster name</th>
<th>Counties included (number of samples)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Americas</td>
<td>Colombia, Peru, Brazil, Panama, Mexico</td>
</tr>
<tr>
<td>Western Pacific</td>
<td>China, Cambodia, Malaysia, Vietnam, PNG</td>
</tr>
<tr>
<td>South East Asia</td>
<td>India, Thailand, Myanmar, Indonesia, Sri Lanka</td>
</tr>
<tr>
<td>Eastern Mediterranean</td>
<td>Pakistan</td>
</tr>
<tr>
<td>African Region</td>
<td>Madagascar</td>
</tr>
</tbody>
</table>
2.6.2.1 Population diversity testing for Hardy Weinberg Equilibrium (HWE) and linkage dis-equilibrium (LD)

Mean number of alleles (MNA) as measure of gene diversity was calculated for each geographical cluster in GeneAEx v.6.5 (Park, 2001). A global test across loci per cluster was implemented in Arlequin v3.5 to calculate mean observed and expected heterozygosities (Excoffier and Lischer, 2010). Observed heterozygosity (H₀) is the number of heterozygotes per locus in a sample from a real population. Expected heterozygosity (Hₑ) in this case relates to sample corrected gene diversity calculated by Nei (1973) as the probability of choosing two non-identical alleles at random at a locus per sub-population (Hedrick, 2005, Nei, 1973).

\[ Hₑ = 1 - \sum_{i=1}^{k} P_i^2 \]

The above equation is used to calculate Hₑ for a locus with multiple alleles. Hₑ is derived by subtracting sum of expected frequency of homozygotes in the population from the total frequency of alleles in the population (Hedrick, 2005).

Under the assumptions of HWE (Hardy Weinberg Equilibrium), the association between alleles at different loci within a population should be random or in other words they should be in linkage equilibrium (LE) (Hedrick, 2005, Page and Holmes, 1998b). If two alleles are linked physically, there is deviation from LE, and they are in linkage dis-equilibrium (LD). LD was measured for multi-allelic population data (MLMT) using two statistics in MULTILOCUS v1.3. First one was Maynard-Smith’s (1993) index of association (Iₐ). Iₐ can be calculated from a comparison between observed (Vₒ) and expected (Vₑ) values of variance in the number of alleles among individuals. Vₑ is calculated on the assumption that alleles are in LE whereas Vₒ is observed from the dataset. Deviation of Iₐ from 0 shows linkage between loci. MULTILOCUS v1.3
generated a p-value for each \( I_A \) value by random permutation assuming infinite recombination.

\[
I_A = \frac{V_0}{V_E} - 1
\]

Presence of LD in a cluster is indicative of a truly clonal mode of propagation, where recombination or genetic exchange is so rare that it cannot break the clonal population structure. However, there are some genetic phenomena that can produce LD in an otherwise recombining population e.g. genetic drift in small populations, population sub-division (Wahlund effect), sampling from biologically and ecologically separated populations and physical linkage of genetic markers used (Page and Holmes, 1998b, Hedrick, 2005, Maynard Smith et al., 1993). MULTILOCUS v1.3, however, can prevent over-estimation of linkage by giving options for pre-defining linkage groups and sub-divisions within clusters.

Since \( I_A \) is reported to be dependent on the number of loci tested (Brown et al., 1980, Maynard Smith et al., 1993), another statistic \( r_{bar-d} \) (or \( r_d \)) was calculated in MULTILOCUS v1.3. Statistic \( r_{bar-d} \) is independent of number of loci tested and calculated under the same theoretical principal as \( I_A \). \( I_A \) and \( r_{bar-d} \) are related across the randomization steps and thus identical p-value is produced for both the statistics by MULTILOCUS v1.3 (Agapow and Burt, 2001).

### 2.6.2.2 Population differentiation or gene flow

Gene flow between any two pairs of sub-populations is one of the mechanisms by which genetic variations can be introduced. The process tends to homogenize gene frequencies between population pairs to limit or prevent differentiation while also increasing heterozygosity of the recipient population. The level of differentiation or gene flow between populations or clusters was assessed using Wright’s F-statistic \( F_{ST} \).
F_{ST} could be calculated as follows,

\[ F_{ST} = \frac{\bar{H}_T - \bar{H}_S}{\bar{H}_T} \]

Where, \( \bar{H}_S \) is the average expected heterozygosity within a cluster/sub-population over loci and \( \bar{H}_T \) is the average heterozygosity in a total population over loci (Hedrick, 2005, Nei, 1977). \( F_{ST} \) conforms to IAM (infinite allele model). Under this model a novel allele is produced at a rate \( u \), with each mutation. It thus, does not permit homoplasy (Kimura and Crow, 1964). However, authors have criticized the suitability of this statistic for microsatellites since it assumes a low mutation rate and removes memory of allelic state prior to mutation (Balloux and Lugon-Moulin, 2002). There is evidence that suggests that size of mutated allele depends on its size prior to mutation.

\( F_{ST} \) for each cluster or sub-population pair was calculated in Arlequin v3.5 (Excoffier and Lischer, 2010) and p-values were generated by randomly permuting genotypes between each pair. The statistic was interpreted according to the guidelines provided by Wright (1978) and Hartl & Clark (1997). They suggest that \( F_{ST} < 0.05 \) indicated little or no differentiation, 0.05-0.15 moderate differentiation, 0.15-0.25 great differentiation, and \( \geq 0.25 \) indicated very great differentiation (only limited gene flow) (Wright, 1978, Hartl and Clark, 1997).

\( F_{ST} \) was also estimated across all sub-populations in context of AMOVA (Analysis of molecular variance) in Arlequin v3.5. The method tested appropriateness of geographical clustering in explaining the overall variance of the data (Excoffier et al., 1992). \( F_{ST} \) is designed to measure sub-division that result from deviation from HWE and thus do not strictly rely on HW allele frequencies (De Meeus et al., 2006).
2.6.2.3 Evolutionary distance

Estimate of evolutionary divergence were also calculated in MEGA v7. Evolutionary distance, the number of substitutions per site separating a pair of homologous sequences as they diverged from their common ancestral sequence, is particularly important measure in molecular evolution and comparative genomics. It is used for a wide range of purposes, ranging from phylogenetic analysis (Saitou and Nei, 1987, Edward and Cavalli-Sforza, 1963), to estimating times of divergence (Kumar & Hedges, 1998; Hedges & Kumar, 2003), the tempo and mode of evolutionary change and functional constraints (Hare and Palumbi, 2003). Evolutionary distance estimation is one of the first steps in high-throughput sequence analysis, errors in these estimates may have wide-ranging consequences on downstream analyses and conclusions (Rosenberg, 2005).
Chapter 3  EPIDEMIOLOGY OF MALARIA IN BANNU REGION

3.1 Introduction

Due to changes in both human and parasite population in Pakistan, monitoring the distribution and burden of *Plasmodium* species is necessary to ensure appropriate treatment, particularly in situations where diagnosis by microscopy or species-specific rapid diagnostic tests is not available (Khattak et al., 2013).

Bannu was considered one of the most malarious area of Indo-Pak region as it is traversed by Innumerable irrigation channels traverse the entire area of the cantonment and its neighborhood and are the main mosquito breeding places. *Anopheles* species (*A. stephensi*, *A. culicifacies* and *A. subpictus*) are found as common vector in the region. Malaria incidence used to peak at the end of August, reach their maximum in October and November, and then decrease rapidly per hospital records. *Plasmodium vivax* and *P. falciparum* are endemic in Pakistan. Bannu (study area) is also reported among the highest malaria incidence areas of Pakistan (Asif, 2008).

Malaria burden increased with displaced population as they belong to malaria endemic regions as Federally Administered Tribal Areas (FATA) was reported to have the highest prevalence of malaria (13.9%) in Pakistan followed by Baluchistan (6.2%), and Khyber Pakhtunkhwa (KP) (3.8%) (DMC, 2017). Cumulative displaced population in 2014 was 435,429 people (males: 114596, females: 137301 and children 183532). Moreover, 73% of the total displaced population are children and women. 17 families have already moved into Bakakhel Camp as of June 22 (FATA disaster managment authority, 2014).

Almost all aspect of malaria epidemiology including community prevalence and age-profile of infection, the incidence and type of disease syndromes and total malaria mortality are affected by malaria transmission intensity (Hay and Snow, 2006, Hay et
Transmission rate also affect the results of malaria control programs because transmission intensity varies geographically. Maps that describe this variation are necessary to identify populations at different levels of risk to compare and interpret malaria interventions conducted in different places, to objectively evaluate options for disease control (Hay et al., 2008).

Incorrect malaria diagnosis and symptomatic diagnosis is the common practice in most endemic countries (Derua et al., 2011). Symptomatic diagnosis results in over-diagnosis of malaria which leads to unnecessary use of antimalarial drugs and possible amplification of disease burden. Misdiagnosis can lead to delay of proper treatment and sometimes death. Therefore, laboratory diagnosis is an important component of case management and control of malaria (Derua et al., 2011).

Malaria diagnostic techniques can be divided roughly into two methods i.e. Direct and indirect methods. Simple microscopy by observing stained slides, rapid diagnostic test (RDT) for parasite antigens and polymerase chain reaction (PCR) for Plasmodial DNA is included in direct diagnostic tests. Indirect tests detect host reaction to infection, like immune-fluorescent antibody test (IFAT), enzyme immunoassay (EIA) and iron pigment detection (Hemozoin) (Seed et al., 2005). World health organization recommends direct visualization of parasites using microscopy or malaria-specific rapid diagnostic test (RDT) before treatment to confirm diagnosis for all clinically suspected malaria cases (CDC, 2017b).

The present study was designed with the main aim of investigating the prevalence of malaria infection among the suspected individuals of Bannu district, Khyber Pakhtunkhwa, Pakistan. In this chapter the applied diagnostic means are compared to assess the specificity and sensitivity of the techniques. Kappa and McNemar’s tests are
applied to find the degree of agreement between different tests. Spearman’s rank correlation analysis was also done to find the correlation among different entities.

3.2 RESULTS

Samples were collected from 2033 malaria suspected individuals visiting malaria model lab, Siddique lab and malaria lab of women and children hospital Bannu. Among them most of the patients were not recommended by doctors for malaria investigation but they came there on their own behalf. A total of 2033 individuals were recruited, of whom 429 (21.1%) were positive for malaria by at least one diagnostic technique.

![District wise origin of patients](image)

**Figure 3.1 District wise origin of patients**

Most of the patients were locals of Bannu region and FR Bannu. Some patients also belong to neighboring districts as diagnostic services of Health facilities of those areas are comparatively not satisfactory (Fig. 3.2).
Figure 3.2 Origin of positive patients shown on Land cover map

Number of *Plasmodium* infected patients belonging to different areas are shown on land cover map (Fig. 3.3). Highest flow of patients was observed in agricultural areas including Bannu, Sarai Naurang, Domel and in areas located near sample collection centers.

3.2.1 Techniques used for the diagnosis of malaria:

Table 3.2 shows the results obtained by analyzing blood samples using various diagnostic techniques. Highest number of samples were analyzed by using microscopy technique, showing 17.66% positive results, followed by RDT with 16.32% positivity results. Highest percentage of positive result of 30.54% obtained by using PCR, although least number of samples were screened through this method. Among PCR positive samples 25.40% were *P. vivax*, 5.14% were *P. falciparum* while no mixed infection was found.
Table 3.1 *Plasmodium* species distribution through different techniques

<table>
<thead>
<tr>
<th>Diagnostic Method</th>
<th>No. of patients screened for each method</th>
<th>No. of malarial infections (% positivity)</th>
<th>No. of total positive (%)</th>
<th>total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microscopy</td>
<td>N=2033</td>
<td>P. vivax 290/2033 (14.26%)</td>
<td>P. falciparum 30/2033 (1.48%)</td>
<td>Mixed 39/2033 (1.92%)</td>
</tr>
<tr>
<td>RDT</td>
<td>N=1618</td>
<td>P. vivax 195/1618 (12.11%)</td>
<td>P. falciparum 30/1618 (1.85%)</td>
<td>Mixed 39/1618 (2.41%)</td>
</tr>
<tr>
<td>PCR</td>
<td>N=311</td>
<td>P. vivax 79/311 (25.40%)</td>
<td>P. falciparum 16/311 (5.14%)</td>
<td>Mixed 0/311 ((0%)*</td>
</tr>
</tbody>
</table>

*Subset of samples screened for PCR had no positive mixed infection by microscopy

### 3.2.2 Locality wise distribution of *Plasmodium* parasite

The highest number of suspected individuals were from Bannu as the laboratories were located in the Bannu region and accessible to residents of the study area. Highest percentage of *P. vivax* (18.4%) infection was recorded from Bannu when compared to other areas. Similarly, more number of *P. falciparum* (2.17%) and mixed (1.7%) infection was recorded from the same region, followed by FATA and Lakki Marwat, while least number was recorded from Karak (Table 3.3).

Table 3.2 Area wise distribution of *Plasmodium* species

<table>
<thead>
<tr>
<th>District</th>
<th>Total No. of patients screened</th>
<th>No. of positive cases (%)</th>
<th>P. vivax N (%)</th>
<th>P. falciparum N (%)</th>
<th>Mixed infections N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bannu (N=1566)</td>
<td>349 (22.3%)</td>
<td>288 (18.4%)</td>
<td>34 (2.17%)</td>
<td>27 (1.7%)</td>
<td></td>
</tr>
<tr>
<td>FATA (N=320)</td>
<td>52 (16.3%)</td>
<td>40 (12.5%)</td>
<td>10 (3.1%)</td>
<td>2 (0.6%)</td>
<td></td>
</tr>
<tr>
<td>Karak (N=19)</td>
<td>4 (21.1%)</td>
<td>2 (1.5%)</td>
<td>0</td>
<td>2 (10.5%)</td>
<td></td>
</tr>
<tr>
<td>Lakki Marwat (N=128)</td>
<td>24 (18.7%)</td>
<td>13 (10.2%)</td>
<td>3 (2.3%)</td>
<td>8 (6.3%)</td>
<td></td>
</tr>
<tr>
<td>Total (N=2033)</td>
<td>429 (21.1%)</td>
<td>343 (16.9%)</td>
<td>47 (2.3%)</td>
<td>39 (1.9%)</td>
<td></td>
</tr>
</tbody>
</table>

Spearman’s correlation analysis was conducted to find correlation between *Plasmodium* species and Localities. The positive correlation was found among *Vivax* infection with District Bannu (0.074%, p=0.004), Lakki Marwat (0.046%, p=0.036) and
FATA (0.05%, $p=0.023$). No significant correlation was found between *falciparum* and Districts. Mixed infection also showed statistically significant positive correlation with District Lakki Marwat (0.082, $p=0.00$) and Karak (0.061, $p=0.006$).

**Table 3.3** Spearman’s correlation analysis between parasite Species and areas

<table>
<thead>
<tr>
<th>Species</th>
<th>Bannu</th>
<th>FATA</th>
<th>Lakki Marwat</th>
<th>Karak</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. vivax</em></td>
<td>0.074** (0.004)</td>
<td>0.050* (0.023)</td>
<td>0.046* (0.036)</td>
<td>-0.016 (0.458)</td>
</tr>
<tr>
<td><em>P. falciparum</em></td>
<td>0.017 (0.44)</td>
<td>0.023 (0.292)</td>
<td>0.0001 (0.98)</td>
<td>-0.015 (0.51)</td>
</tr>
<tr>
<td>Mixed</td>
<td>-0.026 (0.243)</td>
<td>-0.041 (0.066)</td>
<td>0.082** (0.00)</td>
<td>0.061** (0.006)</td>
</tr>
</tbody>
</table>

* Correlation significant at the 0.01 level
** Correlation is significant at the 0.05 level

Most of the individuals visiting the health facilities belonged to Bannu’s two Tehsils i.e., Bannu 1 and Bannu 2. Among 1566 individual, 468 were from Bannu 1 and 1098 were from Bannu 2. Among positive cases 23.5% infected cases were recorded from Bannu 2 and 19.4% were from Bannu 1. Highest prevalence of *vivax* (19.5%), *falciparum* (2.3%) and mixed infection (1.7%) were also found in Bannu 2 while Bannu 1 presented comparatively low values. (Table 3.5)

**Table 3.4** Tehsil wise distribution of *Plasmodium* species

<table>
<thead>
<tr>
<th>Tehsil</th>
<th>Total No. of patients screened</th>
<th>No. positive cases N (%)</th>
<th><em>P. vivax</em> N (%)</th>
<th><em>P. falciparum</em> N (%)</th>
<th>Mixed infections N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bannu 1 N=468</td>
<td></td>
<td>91 (19.4)</td>
<td>74 (15.8)</td>
<td>9 (1.9)</td>
<td>8 (1.7)</td>
</tr>
<tr>
<td>Bannu 2 N=1098</td>
<td></td>
<td>258 (23.5)</td>
<td>214 (19.5)</td>
<td>25 (2.3)</td>
<td>19 (1.7)</td>
</tr>
<tr>
<td>Total N=1566</td>
<td></td>
<td>349 (22.3)</td>
<td>288 (18.4)</td>
<td>34 (2.2)</td>
<td>27 (1.7)</td>
</tr>
</tbody>
</table>

The spearman’s correlation analysis for *Plasmodium* species and tehsils of Bannu District showed statistically significant correlation between *vivax* infection and Bannu
2 tehsil of District Bannu. No other significant correlation value was found among other entities (table 3.6).

**Table 3.5 Correlation of Plasmodium parasites (vivax and falciparum) with two Tehsils of Bannu**

<table>
<thead>
<tr>
<th>Species</th>
<th>Bannu 1</th>
<th>Bannu 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. vivax</em></td>
<td>-0.015 (0.486)</td>
<td>0.076** (0.001)</td>
</tr>
<tr>
<td><em>P. falciparum</em></td>
<td>-0.014 (0.524)</td>
<td>-0.003 (0.909)</td>
</tr>
<tr>
<td>Mixed</td>
<td>-0.008 (0.707)</td>
<td>-0.015 (0.503)</td>
</tr>
</tbody>
</table>

* Correlation significant at the 0.01 level  
** Correlation is significant at the 0.05 level

Out of 2033 samples examined through microscopy, 1618 samples were analyzed using RDT kit while 311 samples were run through PCR. The results showed 266 (16.4%) samples were positive through RDT, out of which 15.94% were also microscopy positive cases while rest of the cases (0.49%) were microscopy negative. 83.18% samples were declared negative through both RDT and microscopy where as 0.37% were RDT negative while microscopically positive.

A total of 95 (30.54%) samples were detected positive through PCR among which significantly high number 64(20.58%) of samples were microscopy negative showing significant difference between both diagnostic procedures. About 9.96% samples were positive by both methods. The same samples when screened through microscopy confirmed positivity for 45 (14.46%) samples while 31(9.96%) could not be detected by using microscopy.
Samples showing negative results for both RDT and microscopy were 1346(83.18%), however, 6(0.37%) were RDT negative but microscopically positive. While 8(0.49%) samples were RDT positive but microscopically negative (Table 3.7 and table 3.8).

**Table 3.6 Comparison of the performance of PCR using microscopy as a standard**

<table>
<thead>
<tr>
<th>PCR</th>
<th>Mic</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neg.</td>
<td>171 (54.98%)</td>
<td>45 (14.46%)</td>
</tr>
<tr>
<td>Pos.</td>
<td>64 (20.58%)</td>
<td>31 (9.96%)</td>
</tr>
<tr>
<td>Total</td>
<td>235</td>
<td>76</td>
</tr>
</tbody>
</table>

**Table 3.7 Comparison of the performance of RDT using microscopy as a standard**

<table>
<thead>
<tr>
<th>RDT</th>
<th>Mic</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neg.</td>
<td>1346 (83.18%)</td>
<td>6 (0.37%)</td>
</tr>
<tr>
<td>Pos.</td>
<td>8 (0.49%)</td>
<td>258 (15.94%)</td>
</tr>
<tr>
<td>Total</td>
<td>1354 (83.7%)</td>
<td>264 (16.3%)</td>
</tr>
</tbody>
</table>

Sensitivity, specificity, PPV and NPV of microscopy were calculated using PCR as the reference test. Sensitivity of the microscopy was 41% with confidence interval (CI) 95% while specificity was 73% (CI=95%). Cohen’s kappa was run to determine agreement between PCR and microscopy for parasite diagnosis. There was moderate agreement between the two diagnostic procedures, $k=0.14$, $p=0.026$. Whereas McNemar test $p=0.069$, shows no significant difference between two raters (diagnostic methods).
PCR and RDT sensitivity, specificity, PPV and NPV were calculated using Microscopy as the reference test. The sensitivity and specificity for PCR analysis was 32% and 79% respectively. The positive predictive value was 41% and negative predictive value was 73%. Kohen’s kappa value was 0.125 (p=0.026) which indicates slight agreement between microscopy and PCR.

Using microscopy as a reference test the sensitivity and specificity of RDT was 79% (CI=95%) and 99% (CI=95%) respectively while positive PPV value was 98% and NPV value was 99%. The kappa value was 0.968 (p=<0.001) which shows the perfect agreement between microscopy and RDT for detection of *Plasmodium* parasite (Table 3.9).

**Table 3.8 Comparing Sensitivity and Specificity of diagnostic methods**

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
<th>McNemar marginal homogeneity test (p value)</th>
<th>Cohen’s Kappa Value (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR</td>
<td>32%</td>
<td>79%</td>
<td>41%</td>
<td>73%</td>
<td>0.069</td>
<td>0.125 (0.026)</td>
</tr>
<tr>
<td>RDT</td>
<td>79%</td>
<td>99%</td>
<td>98%</td>
<td>99%</td>
<td>0.593</td>
<td>0.968 (&lt;0.001)</td>
</tr>
</tbody>
</table>

Spearman correlation analysis test was conducted to examine the association between different species and diagnostic methods.

The Spearman's correlation coefficients between species shows negative relation among *vivax, falciparum* and mixed infection and showed positive relation between species and diagnostic methods i.e. Microscopy, RDT and PCR and also positive relation among these diagnostic methods. The correlation value between *P. vivax* vs. *P. falciparum* (γ= -0.0693, p=0.0018), *P. vivax* vs. Mixed infection (γ=-0.063, p=0.00) are
negatively significant. The statistically positive correlation was found among
*Plasmodium* species and diagnostic techniques (Table 3.10).

**Table 3.9 Correlation between *Plasmodium* species and diagnostic methods and**

**Auto correlation between diagnostic means**

<table>
<thead>
<tr>
<th></th>
<th>P. falciparum</th>
<th>Mix</th>
<th>Microscopy</th>
<th>RDT</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. vivax</td>
<td>-0.0693*</td>
<td>-0.0630*</td>
<td>0.7696*</td>
<td>0.8199*</td>
<td>0.5794*</td>
</tr>
<tr>
<td></td>
<td>(0.0018)</td>
<td>(0.00)</td>
<td>(0.00)</td>
<td>(0.00)</td>
<td>(0.00)</td>
</tr>
<tr>
<td>P. falciparum</td>
<td>-0.0215</td>
<td>0.2378*</td>
<td>0.3043*</td>
<td>0.3538*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.332)</td>
<td>(0.00)</td>
<td>(0.00)</td>
<td>(0.00)</td>
<td></td>
</tr>
<tr>
<td>Mixed</td>
<td></td>
<td>0.3020*</td>
<td>0.3559*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.00)</td>
<td>(0.00)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Correlation significant at the 0.01 level

Among 429 positive patients, 56.4% individuals were showing symptomatic fever and
were found positive for mixed infection while 46.65% individuals with symptomatic
fever were *vivax* positive. Other noticeable percentages was found among individuals
presented with body aches (Table 3.11)

**Table 3.10 Symptom wise distribution of *Plasmodium* species**

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>Total positive cases</th>
<th>P. falciparum (%)</th>
<th>Mixed (%)</th>
<th>P. vivax (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Symptomatic fever</td>
<td>197 (79.9)</td>
<td>15 (31.91)</td>
<td>22 (56.41)</td>
<td>160 (46.65)</td>
</tr>
<tr>
<td>Chill fever</td>
<td>57 (13.3)</td>
<td>3 (6.38)</td>
<td>6 (15.38)</td>
<td>48 (13.99)</td>
</tr>
<tr>
<td>Headache</td>
<td>22 (5.1)</td>
<td>4 (8.51)</td>
<td>3 (7.69)</td>
<td>15 (4.37)</td>
</tr>
<tr>
<td>Body ache</td>
<td>84 (19.6)</td>
<td>8 (17.02)</td>
<td>14 (35.9)</td>
<td>62 (18.02)</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>4 (0.93)</td>
<td>0</td>
<td>0</td>
<td>4 (1.17)</td>
</tr>
<tr>
<td>Vomiting</td>
<td>19 (4.43)</td>
<td>2 (4.26)</td>
<td>3 (7.69)</td>
<td>14 (14.08)</td>
</tr>
<tr>
<td>Anemia</td>
<td>5 (1.16)</td>
<td>0</td>
<td>0</td>
<td>5 (1.46)</td>
</tr>
<tr>
<td>RTI</td>
<td>6 (1.39)</td>
<td>0</td>
<td>0</td>
<td>6 (1.75)</td>
</tr>
</tbody>
</table>
Spearman’s correlation analysis showed statistically significant negative correlation ($\gamma = -0.09$, $p = 0.00$) between *Plasmodium vivax* and body aches while positive correlation ($\gamma = 0.05$, $p = 0.02$) was found between *falciparum* and fever (Table 3.12).

**Table 3.11 Correlation between *Plasmodium* species and symptoms**

<table>
<thead>
<tr>
<th>Species</th>
<th>Fever</th>
<th>Chill fever</th>
<th>Headache</th>
<th>Body ache</th>
<th>Diarrhea</th>
<th>Vomiting</th>
<th>Anemia</th>
<th>RTI</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. vivax</em></td>
<td>-0.013 (0.54)</td>
<td>0.0122 (0.58)</td>
<td>-0.029 (0.18)</td>
<td>-0.09* (0.00)</td>
<td>0.008 (0.71)</td>
<td>0.013 (0.53)</td>
<td>0.005 (0.82)</td>
<td>-0.012 (0.61)</td>
</tr>
<tr>
<td><em>P. falciparum</em></td>
<td>0.05* (0.02)</td>
<td>-0.03 (0.17)</td>
<td>0.017 (0.44)</td>
<td>-0.03 (0.10)</td>
<td>-0.015 (0.49)</td>
<td>-0.008 (0.71)</td>
<td>-0.018 (0.42)</td>
<td>0.226 (0.31)</td>
</tr>
<tr>
<td>Mixed</td>
<td>0.02 (0.29)</td>
<td>0.009 (0.66)</td>
<td>0.01 (0.63)</td>
<td>0.026 (0.22)</td>
<td>-0.014 (0.53)</td>
<td>0.0137 (0.53)</td>
<td>-0.016 (0.46)</td>
<td>-0.021 (0.35)</td>
</tr>
</tbody>
</table>

* Correlation significant at the 0.01 level

Monthly variation of *Plasmodium* species is shown in table (3.13) and Fig 3.5. High frequency of *vivax* is in month of August (30%) followed by July (19.8%), September (17%) and October (14%). Highest peak of *falciparum* was in October (7.6%) followed by May (1.5%) and April (1.2%). No case was reported in August (0%) and September (0%). Fig 3.7 shows that from March to July, the *falciparum* prevalence decreases and it disappears in August and September. After disappearance in the month of August and September, the *falciparum* reaches to the peak in in the month of October. Mixed (*Vivax + Falciparum*) infection shows peak in October (7.6%) and 2nd high prevalence value in April (3.1%). After April, it decreases and disappears in September (0%), then shows second highest peak in October (26%).

Highest peaks of all three types of infections i.e. *Vivax, Falciparum* and Mixed were observed in August (30%), October (7.6%) and October (7.6%) respectively. Lowest prevalence of all three types of infections i.e. *Vivax, Falciparum* and Mixed were
observed in March (3.8%), August & September (0%) and March & September (0%) respectively.

Table 3.13 shows *falciparum* appearance in the month of March, with gradual increase in the following two months of April and May but decline in the month of July and complete disappearance in August and September. Abrupt increase with the highest value of 7.6% was recorded in the month of October.

**Table 3.12 Month wise distribution of *Plasmodium* species**

<table>
<thead>
<tr>
<th>Months</th>
<th>Total</th>
<th><em>P. vivax</em> (%)</th>
<th><em>P. falciparum</em> (%)</th>
<th>Mixed (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>March</td>
<td>132</td>
<td>3.8</td>
<td>0.75</td>
<td>0</td>
</tr>
<tr>
<td>April</td>
<td>324</td>
<td>5.5</td>
<td>1.2</td>
<td>3.1</td>
</tr>
<tr>
<td>May</td>
<td>396</td>
<td>8.6</td>
<td>1.5</td>
<td>0.5</td>
</tr>
<tr>
<td>July</td>
<td>806</td>
<td>19.8</td>
<td>0.74</td>
<td>1.6</td>
</tr>
<tr>
<td>August</td>
<td>110</td>
<td>17</td>
<td>0</td>
<td>0.9</td>
</tr>
<tr>
<td>September</td>
<td>94</td>
<td>14</td>
<td>7.6</td>
<td>7.6</td>
</tr>
<tr>
<td>October</td>
<td>171</td>
<td>14</td>
<td>7.6</td>
<td>7.6</td>
</tr>
<tr>
<td>Total</td>
<td>2033</td>
<td>14.3</td>
<td>1.5</td>
<td>1.9</td>
</tr>
</tbody>
</table>

**Figure 3.3 Seasonal fluctuation of *Plasmodium* infection**
This study was carried out to determine malaria prevalence in clinically suspected individuals at three different laboratories of Bannu. A total of 2033 samples were collected from 1299 male and 734 female individuals. *Plasmodium* species was detected in 21.24% of the samples from male with 2.54% of *Plasmodium falciparum*, 2.16% mixed infection and 16.55% were *Vivax* positive. Samples from females showed 20.84% positivity for *Plasmodium* species including 1.91% *P. falciparum*, 1.50% mixed infection and 17.44% *P. vivax* infection (Table 3.13).

All individuals were divided into three age categories (age in years) i.e. Category-I (0 and above), Category-II (5 and above) and Category-III (25 and above) consisting of 691, 757 and 585 individuals respectively. 20.69% of the *Plasmodium* infected individuals belonged to category-I, 22.46% belonged to category-II and 19.83% belonged to category-III. Among *Falciparum* positive cases 2.60% belonged to Category-I, 2.11% were of category-II and 2.22% were of category-III. Mixed infection comprised of 1.16% of category-I, 1.85% of category-II and 2.91% of category-III. 16.91% of the *Vivax* cases belonged to Category-I, 18.94% and 14.70% belonged to category-II and category-III respectively (Table 3.14).

**Table 3.13 Age and Gender wise distribution of *Plasmodium* species**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Total Positive</th>
<th><em>P. vivax</em></th>
<th><em>P. falciparum</em></th>
<th>Mixed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>276/1299</td>
<td>215/1299</td>
<td>33/1299</td>
<td>28/1299</td>
</tr>
<tr>
<td></td>
<td>(21.24%)</td>
<td>(16.55%)</td>
<td>(2.54%)</td>
<td>(2.16%)</td>
</tr>
<tr>
<td>Female</td>
<td>153/734</td>
<td>128/734</td>
<td>14/734</td>
<td>11/734</td>
</tr>
<tr>
<td></td>
<td>(20.84%)</td>
<td>(17.44%)</td>
<td>(1.91%)</td>
<td>(1.50%)</td>
</tr>
<tr>
<td>Age categories</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(age in years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0&gt;</td>
<td>143/691</td>
<td>117/692</td>
<td>18/691</td>
<td>8/691</td>
</tr>
<tr>
<td></td>
<td>(20.69%)</td>
<td>(16.91%)</td>
<td>(2.60%)</td>
<td>(1.16%)</td>
</tr>
<tr>
<td>5&gt;</td>
<td>170/757</td>
<td>140/757</td>
<td>16/757</td>
<td>14/757</td>
</tr>
<tr>
<td></td>
<td>(22.46%)</td>
<td>(18.49%)</td>
<td>(2.11%)</td>
<td>(1.85%)</td>
</tr>
<tr>
<td>25&gt;</td>
<td>116/585</td>
<td>86/585</td>
<td>13/585</td>
<td>17/585</td>
</tr>
<tr>
<td></td>
<td>(19.83%)</td>
<td>(14.70%)</td>
<td>(2.22%)</td>
<td>(2.91%)</td>
</tr>
</tbody>
</table>
Blood group was asked from patients but many of them have no information about blood group as most of the individuals were illiterate. Among males, highest number of cases were recorded in O positive (22.7%) group followed by A positive (22.2%). Among females O positive have highest percentage i.e. 25%.

**Table 3.14 Relation of Blood group with malaria**

<table>
<thead>
<tr>
<th>Blood group</th>
<th>Positive cases/total Male (%)</th>
<th>Positive cases/total Female (%)</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A+</td>
<td>6/27 (22.2)</td>
<td>0/2 (0)</td>
<td>6/29 (20.7)</td>
</tr>
<tr>
<td>A-</td>
<td>2/4 (50)</td>
<td>-</td>
<td>2/4 (50)</td>
</tr>
<tr>
<td>B+</td>
<td>5/33 (15.2)</td>
<td>1/12 (8.3)</td>
<td>6/45 (13.3)</td>
</tr>
<tr>
<td>B-</td>
<td>1/4 (25)</td>
<td>-</td>
<td>1/4 (25)</td>
</tr>
<tr>
<td>AB+</td>
<td>0/6 (0)</td>
<td>0/2 (0)</td>
<td>0/8</td>
</tr>
<tr>
<td>O+</td>
<td>5/22 (22.7)</td>
<td>1/4 (25)</td>
<td>6/26 (23.1)</td>
</tr>
<tr>
<td>O-</td>
<td>0/2 (0)</td>
<td>0/1 (0)</td>
<td>0/3</td>
</tr>
<tr>
<td>TOTAL</td>
<td>19/98 (19.4)</td>
<td>2/21 (9.5)</td>
<td>21/119 (17.6)</td>
</tr>
</tbody>
</table>

**3.2.3 Month wise malaria data in 2014 as recorded by women and children hospital (WCH) Bannu:**

The data shown in table 3.16 represents the *Plasmodium* species percentage during January-June 2014. Samples analyzed by laboratory technicians through microscopy. Table represent the data for six months, PCR processed samples were collected in July. Highest percentage of *P. vivax* was recorded in June followed by January and March. No mixed infection was recorded in these months while very low percentage of *P. falciparum*, was recorded in April, May and January.
Table 3.15 WCH data collected by analyzed in laboratory

<table>
<thead>
<tr>
<th>Months</th>
<th>Total samples screened</th>
<th>P. vivax (%)</th>
<th>P. falciparum (%)</th>
<th>Mixed infection %</th>
<th>Total +ve %</th>
</tr>
</thead>
<tbody>
<tr>
<td>January</td>
<td>994</td>
<td>15.69</td>
<td>0.20</td>
<td>0</td>
<td>15.89</td>
</tr>
<tr>
<td>February</td>
<td>1536</td>
<td>3.05</td>
<td>0</td>
<td>0</td>
<td>3.05</td>
</tr>
<tr>
<td>March</td>
<td>1484</td>
<td>10.17</td>
<td>0</td>
<td>0</td>
<td>10.17</td>
</tr>
<tr>
<td>April</td>
<td>1452</td>
<td>9.71</td>
<td>0.14</td>
<td>0</td>
<td>9.85</td>
</tr>
<tr>
<td>May</td>
<td>1872</td>
<td>9.51</td>
<td>0.05</td>
<td>0</td>
<td>9.56</td>
</tr>
<tr>
<td>June</td>
<td>1710</td>
<td>17.13</td>
<td>0.17</td>
<td>0</td>
<td>17.31</td>
</tr>
</tbody>
</table>

3.3 Discussion

This chapter aimed to present malaria epidemiology and to assess the efficiency/sensitivity of different diagnostic methods.

Overall 21.1% cases were positive by at least one method. Bannu was considered as one of the most malarious areas of the province (Asif, 2008). Internal displacement of FATA population in 2012 (about 700,000 people from neighbouring North Waziristan) was suspected to have added to the malaria burden in Bannu since FATA regions are known to exhibit highest rates of disease incidence in the country (FATA disaster management authority, 2014, Khattak et al., 2013, Munir et al., 2014). Corroborating previous studies, *P. vivax* infections were more prevalent compared to *P. falciparum* and mixed infections (Razzaq et al., 2014, Khan et al., 2016, Khan, 2014). Dominance of one malaria species over the other is primarily determined by the parasite’s biology and by an area’s climatic and seasonal variations (Checchi et al., 2006). Larger influx of *P. vivax* is possible because true relapses do not occur in *P. falciparum* whereas in *P. vivax* relapses are common due to prolonged survival of *Vivax* hypnozoites in liver cells (Robert and Janovy, 2009, Garnham, 1987, Garnham, 1967).
According to the 2012 national malaria disease surveillance annual report, 9% of Pakistani population were living in high transmission districts (34) with an annual parasitic index (API) ranging from 5 to 28, 20% are living in moderately endemic districts (41 districts) with API ranging from 1 to 5, and 71% living in low endemic districts with an API below 1/1000 population. The national API for the entire country was averaging 1.69, which classifies Pakistan as a moderate malaria endemic country. Malaria mappings show clearly that the highly endemic districts are located mainly in the provinces of Baluchistan, FATA, Sindh, and KP. The lowest malaria incidence was reported in two provinces – Punjab and AJK with combined population of more than 56% of total population of the country (MPR, 2012).

Studies in different districts of Khyber Pakhtunkhwa reported high prevalence of \textit{P. vivax} as compared to \textit{P. falciparum}. In Kohat (\textit{P. vivax} as 86.34% and \textit{P. falciparum} as 13.65%), Buner (\textit{P. vivax} (5.78%) and 1.08% \textit{P. falciparum}), Abbotabad (\textit{P. vivax} as 72.4% and \textit{P. falciparum} 24.1% (Khatta et al., 2012, Muhammad and Hussain, 2003, Idris et al., 2007) are all reported with high \textit{Vivax} prevalence. Malarial infection is more common in rural areas as compared to urban regions of the study area (Khan et al., 2016, Khan et al., 2013a). Agricultural activities, irrigation system, poor sewage management, ignorant behavior towards protective measures, sleeping habit are the factors contributing to increase prevalence in rural population.

Other studies in different parts of Pakistan also reported high prevalence of \textit{P. vivax} (6%-71%) as compare to \textit{P. falciparum} (0.5%-37%) (Yar et al., 1998, Jan and Kiani, 2001, Mehmood, 2005, Yasinzai, 2008). The highest burden of \textit{vivax} malaria was reported from Khyber Pakhtunkhwa and the Federally Administered Tribal Areas, illustrating the need for greater programmatic and health system strengthening in these regions (Murtaza et al., 2009). Pakistan is almost in the middle of malaria belt around
the globe among tropical and subtropical countries where majority of population is living in rural areas (Soomro et al., 2009). In towns, the defaulted sewerage system, stagnant water, improper dumping of garbage contributes to the spread of malaria.

In our study, *P. vivax* cases peaked in the summer month (August) while *P. falciparum* and mixed infections in winter (October). Similar distribution trends are known to exist in certain areas of neighboring Afghanistan that share similar climatic settings (Zakeri et al., 2006, MOH, 2010, WHO, 2011b). Generally, in Pakistan *P. falciparum* transmission starts in the summer monsoon (July) when the temperature and humidity is optimum and it prevails until the end of the year when the temperature falls below the critical value (December). *P. vivax* usually observes two transmission peaks, one is its early transmission period during the wet months of spring (probably facilitated by true relapses) and the other is with the *P. falciparum* (monsoon) (Bouma et al., 1996a, MOH, 2010, Asif, 2008, Khan et al., 2006). The seasonal abundance of vector as irrigation system and rain fall pattern of Pakistan provides ideal conditions for mosquito breeding. (MPR, 2012, Chaves and Koenraadt, 2010). In District Bannu number of malaria cases rises towards ends of August, maximum in October and November then decreases onward (Dogra, 1938).

Similar findings were reported from different parts of KP including Karak, shangla, Buner, Lower Dir, Mardan, Peshawar (Daud et al., 2014, Rahman et al., 2017, Muhammad and Hussain, 2003, Zeb et al., 2015, Majid et al., 2016), FATA (Khan et al., 2016) and other regions (Amodu et al., 1998, Lathia and Joshi, 2004). The recent malaria cases were reported from Bannu, Hangu and Charsadda while no malaria case was reported from nine districts including Abbottabad, Haripur, Kohistan, Mansehra, Battagram, Torghar, Lower Dir, Upper Dir and Shangla (Associated Press of Pakistan, 2016).
Possible reasons for this high prevalence of malaria may be, raining during monsoon and favorable temperature providing suitable conditions for the growth of mosquitoes thus increased in malaria cases (Nizamani et al., 2006, Macdonald, 1956, Hussain et al., 2013, Mehmood, 2005, Farogh et al., 2009, Jahan and Sarwar, 2013, Strickland et al., 1987, Yaseen and Ali, 2015). Low temperature and dry season during winter hinders the sporogonic development (Trape et al., 1992). Currently climatic change, behavior of different vectors and _Plasmodium_ parasite according to Schmalhausen’s law is under consideration for understanding seasonality of malaria in certain parts of the world (Basurko et al., 2011).

Yaseen & Nafisa, (2015) reported same ratio of _P. vivax_ and _P. falciparum_ cases in post monsoon months and winter (October- February) (Yaseen and Ali, 2015), while seasonal variation of both species during post-monsoon and dry season has been reported (Anya, 2004, Zacarias and Andersson, 2010). _P. falciparum_ emerged as predominant species during 1981-1985 (Rafi et al., 1994). Outbreaks of _P. falciparum_ malaria frequently occur, particularly during the rainy season (April–September) in India (Dev and Dash, 2007). Immigrants from Afghanistan (_P. falciparum_ endemic) are responsible for its spread in Southern Punjab, Baluchistan and Sindh provinces (Atif, 2009).

The dominance of one malaria species over the other at a certain period might vary from one area to another not only depending on climatic and seasonal factors but also owing to variation in geographical localities (Checchi et al., 2006). In the present study, the infection rate of _P. vivax_ was higher than _P. falciparum_, because there seems to be no second exothermic cycle and true relapses do not occur in _P. falciparum_ whereas in _P. vivax_ relapses occurs due to prolonged survival of _Plasmodium vivax_ hypnozoites in liver cells (Garnham, 1967, Garnham, 1987) and these relapses are also responsible for
outbreak of the disease (Feighner et al., 1998, Chadee et al., 1992). Moreover the longevity of *P. falciparum* in man seldom exceeds one year and *P. vivax* usually die-out within three years (Bruce-Chwat, 1980). It is observed that controlled *P. falciparum* transmission led to an increase in relapsed *P. vivax* cases because of suppressive effect of *P. falciparum* on *P. vivax* prevalence (Rowland, 2001).

Unlike the findings of our study, age has earlier been associated with malaria acquisition (Dondorp et al., 2008, Ferede et al., 2013) where children ≤ 5 years are shown to carry a comparatively higher risk (Bodker et al., 2003, Brooker et al., 2004, Peterson et al., 2009). The acquired immunity is both exposure- and age-dependent, and the older children are likely to have developed some degree of immunity because of repeated infections (Bodker et al., 2006, Woyessa et al., 2013). There also exists a possibility of extended exposure due to inattentiveness of parents/guardians towards protective/treatment measures (Peterson et al., 2009). Further, we documented no significant association with gender. Although, several earlier studies in Khyber Pakhtunkhwa and elsewhere prove males to be at a higher risk of malaria compared to females (Khan et al., 2013a, Awan and Jan, 2008a, Razzaq et al., 2014, Awan and Jan, 2008b, Rahman et al., 2017, Irshad et al., 2013, Daud et al., 2014, Sahar et al., 2010, Rehman et al., 1994, Kondrachine, 2008, Khan et al., 2016) suggesting increased exposure to mosquito bites since males are more likely to work outdoors and are not traditionally well-covered as females (usually veiled) (Khan et al., 2006). Cultural and social norms might have stemmed low influx of female patients to health care facilities.

A comparison of age-specific parasite rates of malaria in Afghan refugees and nearby local population at Karachi shows that Afghan refugees are susceptible to malaria even in later age-groups while infections in the local population were limited to younger age-
groups. Similarly, prevalence of malaria over the years is much higher in Afghan refugees than in the local population which is due to the low herd immunity in Afghan refugees (Suleman, 1988b). The children have low immune system as compared to the adults (Khan et al., 2016).

In contrast to earlier studies from Buner (KP) and (Muhammad and Hussain, 2003) and different areas of Baluchistan (Yasinzai and Kakarsulemankhel, 2003, Yasinzai and Kakarsulemankhel, 2004, Yasinzai and Kakarsulemankhel, 2007), in the present study mixed infections (1.92%) were detected by microscopy only, unfortunately conclusive statement couldn’t be given about authenticity of these positive mixed infection as PCR was not carried out for these samples. Mixed infections are frequently detected through PCR as microscopy usually fails to detect mixed infections due to presence of higher numbers of parasites of one species relative to the other (Singh et al., 1999a).

There is no report from Pakistan for detection of mixed infection by microscopic examination, although high percentage of mixed malaria infection was reported through PCR. Mixed infection rate of 6.5 %, 22%, and 23.5 % was found in Iran, Afghanistan and Pakistan respectively by PCR (Zakeri et al., 2010a). Due to poor quality microscopy practices, mixed infections are rarely diagnosed and reported, less number of mixed infection (0.77%) was reported from endemic areas of Iran while no mixed infection was reported from Afghanistan and Pakistan through the same technique (Zakeri et al., 2010d). A study conducted in India reported 45.5% of mixed infections by PCR assay while no case of mixed infection was detected through microscopy (Gupta et al., 2010). The main challenge is the detection of \textit{P. falciparum} parasitaemia when it is superimposed by \textit{P. vivax}. In such circumstances, additional tests such as PCR or ELISA are essential for an accurate diagnosis (Noedl et al., 2006). A study conducted in Flores, Indonesia, reported more sensitivity of PCR in comparison with
microscopy in case of sub microscopic infections in low transmission areas (Kaisar et al., 2013).

In the present study, no mixed infection was found among PCR processed samples either through PCR or Microscopy as collection was done in July, during this month *falciparum* infection does not exist except those having previous dormant infection (Bouma et al., 1996a). During routine laboratory investigation, no mixed infection was found through microscopy in previous months as *falciparum* is prevalent from September to December in the study area. Mixed malaria species infections are often underestimated in surveys from Asia (3%) therapeutic studies showed that the estimated rate is much higher (up to 30%) (Mayxay et al., 2004), 29% in Africa (Rubio et al., 1999) and Venezuela (Postigo et al., 1998) and 5% in Pakistan (Khatoon et al., 2010).

Absolute parasite identification is an important factor for eradication of any disease. There is lack of accurate diagnostic procedures for *Plasmodium* identification in many malaria endemic regions, where dependence on the appearance of clinical signs and symptoms alone are insufficient and vague pointers of specific disease (Fransisca et al., 2015). In the current study, we also compare the results of different diagnostic techniques used for clinically malaria suspected individuals in malaria endemic region. Microscopy positive cases were 17.66% including 14.26%, 1.84% and 1.92% were *P. vivax*, *P. falciparum* and mixed infection respectively. One of the actual challenge for microscopy is mixed infection as similarities exist between developmental stages of different *Plasmodium* species. Some individuals were detected as *vivax* positive through microscopy while co-infection was indicated by RDT. In accordance with this study, in Thailand patients (∼8%) treated for *P. vivax* malaria are later found to be co-infected with *P. falciparum* (Mayxay et al., 2001).
Among 1618 samples screened through RDT, 264 (16.32%) were detected positive for *Plasmodium* infection among which percentage of *vivax*, *falciparum* and mixed infection was 12.11%, 1.85% and 2.41% respectively. Some microscopy missed cases were identified by RDT which might be individuals who had been treated but antigen remain persistent in them (Moody, 2002) but possibility of false positive detection by RDT remain exist.

RDT shows more sensitivity to *falciparum* and mixed parasitaemia as compared to non-*falciparum* infection which may be attributed to longer persistence of *falciparum* antigen (HRP-2) after treatment or resolution as compared to other Plasmodial antigen (pLDH) which quickly disappear after treatment (Bell et al., 2005, Hopkins et al., 2007).

WHO recommends RDTs and microscopy as primary methods for diagnosis of clinically suspected malaria in all epidemiological settings, including low transmission areas like Khyber Pakhtunkhwa (WHO, 2014b). In our study, among 1618 samples screened through RDT, 266 (16.4) were detected positive for *Plasmodium* infection. RDT presented satisfactory sensitivity (79%) when compared to microscopy. The results of RDT was also compared with microscopy (gold standard), RDT shows perfect agreement with microscopy kappa value (k =0.968) (Landis and Koch, 1977). These results match with the results of Kim *et al* (2013) showing greater agreement between RDT and microscopy with high kappa value (0.896-0.991) (Kim et al., 2013). The specificity, PPV and NPV values of RDT against microscopy were 99%, 98% and 99% respectively. High positive predictive value recorded in this study is higher than previous studies that was in the range of 64% to 71% (Ashton et al., 2010, Singh et al., 2010) and 90.2% by Ameh *et al* (Ameh, 2012). The finding of the present study is similar to the study conducted in Nigeria where the antigen based method has a better
correlation with both the gold standard i.e. microscopy and the clinical settings. The antibody based method as anticipated showed good level of sensitivity but, highly unspecific (Azikiwe et al., 2012). These results provide basis for use of RDT as screening tool and on the basis of its results the clinicians can proceed with the treatment of malaria positive individuals.

Overall findings from the study provide reasonable basis for use of RDT as a screening tool in field and for clinicians to proceed with timely treatment of malaria patients. Studies also support the superior performance of RDT compared to microscopy in routine clinical settings and especially in remote locales where medical units face deficit in resources like contained labs, electricity, trained microscopists etc (Ohrt et al., 2002, Erdman and Kain, 2008). RDTs can serve as a cost-effective method for clinical diagnosis, mainly due to improved treatment and health outcomes for non-malaria febrile disease (Shillcutt et al., 2008). Incorrect diagnoses are a common observation in labs like the ones selected in our study, largely due to huge numbers of patients availing for free diagnosis. Furthermore, we observed that many of the patients visited the labs for diagnosis without being referral by clinicians.

Similar finding was reported in previous studies with minor differences between results of microscopy and RDT which may be due to the use of antimalarial drugs. Rapid device did not detect *P. vivax* trophozoites while seen under microscope were due to use of anti-malarial drugs that kill the parasite and are no more able to secrete *Plasmodium* lactate dehydrogenase enzyme (PLDH) as secreted by the living forms only (Palmer et al., 1998, Zeb et al., 2015).

Operative study comparing traditional blood smear to rapid antigen capture test demonstrates that the RDT, performed in a routine clinical setting is superior to a single
set of Giemsa-stained blood smears for quickly evaluating a patient for malaria. Importantly, the rapid antigen capture assay had a 100% NPV for *P. falciparum* malaria. Light microscopy can routinely detect parasitemia level as low as 40 parasites/μl, and experienced microscopists can detect as low as 5-10 parasites/μl of blood (WHO, 2009).

Sensitivity and specificity of RDTs are measured on the basis of HRP-2, pLDH (parasite lactate dehydrogenase) and PL-aldo (*Plasmodium* aldolase) antigens targeted by RDT, HRP-2 is secreted by *P. falciparum* while later are secreted by another species (Moody, 2002). For detection of low parasitaemia level there should be high sensitivity and specificity of the RDT. The decrease specificity from WHO recommended values can be a result of drug intake before diagnosis which clears the parasitaemia from circulating blood however HRP-2 (*Falciparum* antigen) remain circulates in blood even post treatment (Chinkhumba et al., 2010).

Different factors related to parasite and host can also influence the RDT performance while its sensitivity is influenced by density of parasitaemia (Shakya et al., 2012, Kim et al., 2008). Variation in antigen structure (Baker et al., 2005, Lee et al., 2006), gene deletion (Gamboa et al., 2010) and prozone effect (prevent the stimulation of antigen-antibody complex which is essential for visibility of test lines, specifically associated with HRP-2 antigen) (Gillet et al., 2009, Luchavez et al., 2011) are the reported factors for false negative results. Previous studies reported 58% sensitivity of RDT in case of ≤100/μL malaria parasite concentration, sensitivity drops to almost zero at concentration of 0.001% (≤50/μL) where microscopy also showed negative result (Maltha et al., 2011).
Incorrect malaria diagnosis leads to increased illness and mortality, drug resistance due to administration of wrong drugs and ignorance of causes of fever such as bacterial infection, leads to prolonged and ineffective treatment. As in study area PCR cannot be used as a routine diagnostic method, chances of correct diagnosis should be increased by use of RDT along with microscopy (Ngasala et al., 2008, de Oliveira et al., 2009, Hopkins et al., 2008).

Among PCR processed samples, 30.54% (95) were positive for Plasmodial infection, among which 25.40% and 5.14% were *vivax* and *falciparum* positive respectively. Although all quality standards were assured during PCR experiment in this study, there exist a risk for contamination in such a sensitive technique. In the present study, nested PCR detect 20.58% (64 of 95) more cases which were microscopically declared negative. Among *P. falciparum* positive cases, ten were diagnosed as *P. vivax* infection through microscopy but through PCR they were detected *vivax* negative. This shows either poor quality of the microscopy in the study but the risk of contamination in PCR cannot be excluded.

In the present study, microscopy displayed poor sensitivity when compared to rRNA PCR. The results of microscopy diagnostics were compared with PCR (Gold standard). The microscopy shows slight agreement as kappa value (k =0.14) with PCR with the sensitivity and specificity of Microscopy evaluated against PCR (gold standard). The sensitivity, specificity, PPV and NPV values were 41%, 73%, 32% and 79% respectively. Similar results were found in previous studies conducted in Ethiopia, microscopy shows low sensitivity (52%) when compared with Nested PCR for *P. falciparum* and *P. Vivax* identification (WHO, 2014a). Studies suggest that microscopy, when compared to molecular diagnostic methods like PCR; often exhibit considerable discrepancies (usually showing lower sensitivity to mixed infections), especially in
submicroscopic infections (Mayor et al., 2012, Arango et al., 2013, Kaisar et al., 2013); (Ohrt et al., 2002). Parasite densities measured by microscopy correlate with parasite gene copy number in quantitative PCRs and studies indicate that in submicroscopic infections lower copy numbers are expected in the low transmission settings than those in the high transmission. These deviations eventually impact diagnostic outcomes (Lo et al., 2015, Okell et al., 2012b). Moreover, mixed infections pose challenges to microscopists since similarities exist between developmental stages of different *Plasmodium* species. Microscopy and RDT are prone to miss infections with density less than 100 parasites/μL, while the detection limit of PCR is generally < 5 parasites/μL (Cordray and Richards-Kortum, 2012, Vasoo and Pritt, 2013). Lack of expertise and factors like lack of standard good quality blood films also often contribute to such incongruities (Rantala et al., 2010, Ohrt et al., 2002). On the other hand, PCR will rarely miss microscopy positives due to poor DNA quality, poor quality standards in techniques or missed priming due to mutations (Coleman et al., 2006a). Although studies demonstrate superior performance of PCR traditional diagnostics means in field (Haghdoost et al., 2006, Zakeri et al., 2010c); (Rodulfo et al., 2007). PCR cannot be used as a routine diagnostic tool due to its technologically advanced lab requirements and expenditures (Ngasala et al., 2008, de Oliveira et al., 2009, Hopkins et al., 2008).

For collecting more accurate epidemiological data from unapproachable areas of world, PCR is more sensitive technique in combination with filter paper blood spot collection as compared to other methods (Singh et al., 1999b). The high sensitivity of PCR than microscopy for parasite detection is attained by nested method and previous results confirm these observations that there is a sharp cut-off point for the nested PCR assay resulting in an all or none detection of parasites (Snounou et al., 1993b).
Sensitivity of genus specific PCR amplification is more than species specific amplification as for primers targeting at least two ssrRNA genes are present in Plasmodial genome (McCutchan et al., 1988, Gunderson et al., 1987, Li et al., 1997). Primers for identification of *Plasmodium* species i.e. *P. vivax* and *P. falciparum* are specific only for expressed C-type ssrRNA genes of both species (McCutchan et al., 1988, Li et al., 1997) thus, failure of PCR in specie identification might be due to low parasitemia (Kawamoto et al., 1996).

In opted laboratories, the technicians were trained for microscopy but still they lack the professional skills for correct diagnosis of species identification. Incorrect diagnosis is a common practice in these labs due to patient’s overflow as among these three labs, two were government facilities where patients were not charged for malaria test so many people visited the lab just for no reason or without any symptoms. Another reason for overflow is the location of the facilities, located in the center of the study area.

### 3.4 Conclusion

1. Malaria infections in Pakistan are largely credited to *P. vivax* but *P. falciparum* and mixed species infections are also prevalent. In addition, regional difference in the prevalence and species composition of malaria is high.

2. The data obtained was checked according to the gender, age, season and species wise in the human population of Bannu. Males (up to 24 years’ age) are more susceptible to malaria as compared to females. The seasonal prevalence pattern is almost similar to the previously reported studies conducted in the study area, country and in other regions of world, *Plasmodium vivax* and *Plasmodium falciparum* are the prevalent species in District Bannu with *P. vivax* being the most prevalent.
3. In this study, we followed the questionnaire based data collection from different patients through local health facilities and diagnostic labs. It is concluded that load of malaria is high in rural centers as compared to urban centers where large population belonged to lower class from economic point of view.

4. RDT provided good test sensitivity, specificity, positive and negative predictive values as well as inter-procedure agreement for detecting parasite as compared to Microscopy. RDT can become the first screening test for the diagnosis of malaria in the laboratory.

5. Current evidence indicated that no single method for the diagnosis of malaria is perfect and none of them be a stand-alone accurate and effective diagnostic criterion. Both symptoms/signs and laboratory-based malaria diagnostic methods should be employed for an accurate and effective diagnosis of malaria. Various factors including malaria endemicity, the urgency of the diagnosis, transmission pattern, the experience of the health worker, effectiveness of the health care system and available budget resources greatly affect the priority of any of the malaria diagnostic method.

3.5 Recommendations

1. A well designed, longitudinal and multi-centered study is suggested to explore the seasonality of malaria and its drivers in our environment.

2. Accurate knowledge of this common illness can help the community and the malaria control program authorities to focus on eradication of malaria. To control the disease, awareness about the disease, early treatment and preventive measure are required.
3. Evaluation and comparison of different environmental factors and climate or vector drivers for malaria.

4. Awareness campaigns to encourage the use of repellents, protective clothing, screening, bed-nets and other forms of personal protection against the bite of mosquitoes are important and widely recommended (Valecha et al., 1996).

5. The community should be informed and educated about the importance of early diagnosis and prompt treatment with effective antimalarial.

6. Improvement in the quality of microscopic techniques in clinical laboratories and provision of PCR and RDT to those health facilities are needed where microscopy standard is not up to the mark (Cuadros et al., 2015).
Chapter 4  RISK FACTORS ANALYSIS FOR MALARIA

4.1 Introduction

Among the Pakistani population, roughly 60% live in malaria endemic regions (Williams and Meek, 2011). After eradication efforts in the 1960s, malaria surged back to an epidemic level in the 1970s. In recent years, an increase in malaria can be partially attributed to floods that affected approximately 20 million people in over 60 districts (Williams and Meek, 2011). About 0.5 million cases of malaria and close to fifty thousand deaths are attributed to malaria in Pakistan per year (Mukhtar, 2006).

Pakistan being a tropical and agricultural country where majority of population is poor and lives in the rural areas. The incidence of Increase of malaria cases in this part of the world is due to several factors including increased poverty, environmental deterioration and particularly the spread of chloroquine resistance. Warmer autumns which favor prolonged transmission and a chronic decline in vector control activities are also contributing in the spread of malaria (Rowland et al., 1997b, Tasawer et al., 2003). Similarly, the prevailing extensive agricultural practices, an expansive irrigation network, and the monsoon rains act together to promote a favorable environment for malaria transmission in many areas of Pakistan (Ghanchi et al., 2011).

The study area (Bannu) of Pakistan consists of agricultural bushes where better sites for malaria vectors growth are linked with the crop cultivation, increasing subsequent infection risks. The poor hygienic condition, no use of antimalarial sprays, use of irrigated land, sharing the house with livestock, improper diagnosis, high temperature of the area and load shading also play a key role for the spread of malaria in the Bannu District (Khan et al., 2013a).
Like other infectious diseases malaria has very complex epidemiology regarding the parasites, its vectors, the human hosts and the environment (van der Hoek et al., 1998). For designing an effective control and prevention strategy it is essential to focus on the relationship among malaria transmission, human related factors and climatic variables to reduce the burden and transmission intensity of the disease. Human related factors are known to intensify the range of climatic factors. In malaria endemic regions, risk of infection to human population varies with geographical conditions of the areas within the same country. Malaria incidence, transmission rate and vector distribution can vary between neighboring villages and small settlements within same village, due to variation in risk factors (Lindsay et al., 1990, Drakeley et al., 2003, Snow et al., 1997, Reyburn et al., 2005).

Apart from rainfall and river-flow velocities other factors are also identified as risk factors such as utilization of control measures (van der Hoek et al., 1998), age and gender (Mendis et al., 1990), human migration (Klinkenberg, 2001), as well as type and location of housing (Gamage-Mendis et al., 1991, Gunawardena et al., 1998, van der Hoek et al., 1998). Because of geographical and chronological variation of malaria transmission it is essential to analyze the basic risk factors, so that control efforts can be targeted to the high-risk areas (Klinkenberg et al., 2004).

It is epidemiologically proved that in low transmission areas the malaria infection is restricted within communities. Malaria risk varies from person to person due to individual characteristics (Ayele et al., 2012). In areas with stable transmission, age (<5 years) is reported as a high risk of malaria morbidity and mortality (Snow et al., 2005). Kreuels et al. analyzed the distribution of sex, ethnic background, infant’s birth season, sickle cell trait (HbAS compared with HbAA), mother’s education and occupation, mother’s knowledge of malaria transmission and use of protective measures (Kreuels
et al., 2008). Malaria risk factors studies mostly failed to examine the wide range of both household and individual level factors as omission of several factors leads to unsatisfactory results (Mauny et al., 2004). Further research is needed to clarify the different effects of socioeconomic and environmental risk factors on severe malaria occurrence.

The current study was designed to examine the prevalence of malaria infection and associated risk factors in Bannu District, where transmission of malaria is perennial. The disease is endemic in different parts of Khyber Pakhtunkhwa, including the study site. This study was aimed to recognize the possible risk factors of malaria, its relationship with infection and exposure which will be helpful in the prevention and control of the disease in this area. Individual and household characteristics (fever history, previous malarial illness and protection from mosquitoes, construction material, vicinity to mosquito breeding sites and socio economic status) were studied. The relationship of these factors to the number of malaria cases was analyzed.

4.2 Materials and method;

4.2.1 Study site and design:

The present study was carried out in three laboratories of Bannu District (32° 43' - 33° 06' N; 70° 22' - 57' E), Khyber Pakhtunkhwa Province. Rivers Kurram and Gambila passing through Bannu serve the area for irrigation and provides breeding grounds for Anopheles (Malaria vector). The study area has two rain season, in March and summer moon soon during July and August (MOHP, 2017).

4.2.2 Laboratory method

Three diagnostic procedure (Microscopy, RDT, PCR) were used as described in chapter 2.
4.3 Risk factors data

The risk factors questionnaire data collected individual and household information (Appendix B). The data from each individual were taken either from patient or guardian of the patient. Questionnaire recorded information about living area, previous illness, presence of domestic animals, housing type, proximity to vector breeding site, use of preventive methods (e.g., coils, house spraying), bed net use socioeconomic status, sleeping habit, accessibility to health facility, intake of antimalarial drugs etc.

4.4 Statistical analysis of risk factors data

A database was created in Ms Excel and transferred to Stata 14. Univariate and multivariate logistic regression model was used, to investigate whether socio-economic and demographic factors as a risk factors of malaria prevalence of infection and exposure in the studies population. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were computed. Risk factors were analyzed for three diagnostic techniques separately. Distribution maps of *Plasmodium* species in study areas and nearby districts were produced by using ARC GIS. Data was paired with positive and control cases through stata 14 (age and gender-wise pairing of positives). To determine the effect of each exposure variable on the outcome variable (prevalence of infection) univariate analysis was performed. Any exposure variable significant with $p \leq 0.05$ in univariate analysis was used in multivariate analysis (Wald test). The final multivariate modal includes only significant exposure variable with $p \leq 0.05$.

4.5 Results

Total 2033 samples were analyzed for the detection of *Plasmodium* infection.
Table 4.1 Detail and division of potential risk factors for malaria in the study area

<table>
<thead>
<tr>
<th>Individual and Behavioral risk factors</th>
<th>Household/residential risk factors</th>
<th>Vector control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>Locality</td>
<td>Insecticide residual spray (IRS)</td>
</tr>
<tr>
<td>Sex</td>
<td>Distance of water body</td>
<td>Use of bed nets</td>
</tr>
<tr>
<td>Education level</td>
<td>House construction</td>
<td></td>
</tr>
<tr>
<td>Fever history</td>
<td>Windows glazing</td>
<td>Personal Mosquitoes Protection</td>
</tr>
<tr>
<td>Treatment history</td>
<td>socio-economic status (SES)</td>
<td></td>
</tr>
<tr>
<td>Sleeping habit, Blood group, General Health, Symptoms</td>
<td>Animals ownership</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Access to health providers</td>
<td></td>
</tr>
</tbody>
</table>

4.5.1 Univariate analysis for Microscopy positive samples

A full list of factors investigated for association with the prevalence of *Plasmodium* infection (detected by Microscopy) are shown in table. 2033 individuals including 359 positive cases and 1674 controls, were matched by gender and age categories to derive 359 matched pairs in Stata Statistical Software: Release 13 (StataCorp LP, 2013). Using this case-control data, risk factor assessment was performed through univariable and step-wise multivariable conditional logistic regression in Stata v13.

To find the association with potential risk factors and disease univariate odds ratios (OR) were estimated by logistic regression with confidence interval (95%). The univariate analysis showed that satisfactory health condition (OR=0.24, P=0.00, 95% CI), sleeping inside the room (OR=0.62, P=0.002, 95%CI), presence of health care facility in living area (OR=0.06, P=0.006, 95%CI) and the persons economically belongs to upper middle class (OR=0.68, P=0.04, 95% CI) are statistically significant for reducing risk of malaria.
4.5.2 Multivariate analysis for Microscopy positive samples

The details of Multivariate logistic regression analysis for risk factors and prevalence of *Plasmodium* infection detected by microscopy are given in table 4.2. The multivariate regression model showed a significant effect of apparently satisfactory health condition (OR=0.24, P=0.00, 95% CI), sleeping inside the room (OR=0.69, 0.02, 95% CI) and health care facility in living area (OR=0.61, P=0.003, 95% CI) in reducing risk for malarial infection. This model showed these factors as a protective factor as having odds ratio less than 1 (OR<1).
### Table 4.2 Risk factors for microscopy

<table>
<thead>
<tr>
<th>Variable</th>
<th>Odd ratio (confidence interval)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age group (compared to &lt;5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;5</td>
<td>1 (95%)</td>
<td>1</td>
</tr>
<tr>
<td>&gt;20</td>
<td>1 (95%)</td>
<td>1</td>
</tr>
<tr>
<td>Sex (female compared to male)</td>
<td>1 (95%)</td>
<td>1</td>
</tr>
<tr>
<td>Literacy (compared to illiterate)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary &amp; above</td>
<td>1.330619 (95%)</td>
<td>0.325</td>
</tr>
<tr>
<td>Secondary &amp; above</td>
<td>.8828151 (95%)</td>
<td>0.464</td>
</tr>
<tr>
<td>Duration of previous malaria illness (compared to no illness)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;15 Months</td>
<td>1.056139 (95%)</td>
<td>0.645</td>
</tr>
<tr>
<td>&gt;15 Months</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Previous illness</td>
<td>0.9666325 (95%)</td>
<td>0.821</td>
</tr>
<tr>
<td>Recurrent malaria (No/yes)</td>
<td>1.913154 (95%)</td>
<td>0.144</td>
</tr>
<tr>
<td>Fever history</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>General health (compared with poor health)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weak</td>
<td>.8216903 (95%)</td>
<td>0.35</td>
</tr>
<tr>
<td>Satisfactory</td>
<td>.2355209 (95%)</td>
<td>0</td>
</tr>
<tr>
<td>Domestic animals (No/yes)</td>
<td>1.312242 (95%)</td>
<td>0.071</td>
</tr>
<tr>
<td>Screening against malaria (No/yes)</td>
<td>1.168308 (95%)</td>
<td>0.378</td>
</tr>
<tr>
<td>House type (compared with Mud)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concrete</td>
<td>.7988062 (95%)</td>
<td>0.138</td>
</tr>
<tr>
<td>Tent</td>
<td>1.671007 (95%)</td>
<td>0.322</td>
</tr>
<tr>
<td>Water reservoir in vicinity (No/yes)</td>
<td>1.44321 (95%)</td>
<td>0.179</td>
</tr>
<tr>
<td>Sleeping habit (compared to outside)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inside</td>
<td>.6256993 (95%)</td>
<td>0.002</td>
</tr>
<tr>
<td>Blood group (compared to A blood group)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>.875 (95%)</td>
<td>0.858</td>
</tr>
<tr>
<td>AB</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>O</td>
<td>.65625 (95%)</td>
<td>0.573</td>
</tr>
<tr>
<td>Health care facility in living area (No/yes)</td>
<td>.6466535 (95%)</td>
<td>0.006</td>
</tr>
<tr>
<td>Anti-malarial drugs intake (No/yes)</td>
<td>1.057517 (95%)</td>
<td>0.708</td>
</tr>
<tr>
<td>Socio economic status (compared with lower class)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lower middle</td>
<td>.8449726 (95%)</td>
<td>0.368</td>
</tr>
<tr>
<td>Upper middle</td>
<td>.6766382 (95%)</td>
<td>0.046</td>
</tr>
<tr>
<td>Upper class</td>
<td>.5713833 (95%)</td>
<td>0.09</td>
</tr>
<tr>
<td>Symptomatic fever (No/yes)</td>
<td>.8944738 (95%)</td>
<td>0.455</td>
</tr>
<tr>
<td>Chill fever (No/Yes)</td>
<td>1.48448 (95%)</td>
<td>0.078</td>
</tr>
<tr>
<td>Headache (No/yes)</td>
<td>1.053107 (95%)</td>
<td>0.872</td>
</tr>
<tr>
<td>Body ache (No/yes)</td>
<td>.7667148 (95%)</td>
<td>0.123</td>
</tr>
<tr>
<td>Diarrhea (No/yes)</td>
<td>.6629128 (95%)</td>
<td>0.527</td>
</tr>
<tr>
<td>Vomiting (No/yes)</td>
<td>.8425439 (95%)</td>
<td>0.613</td>
</tr>
<tr>
<td>Anemic (No/yes)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Respiratory tract infection (No/yes)</td>
<td>.5932011 (95%)</td>
<td>0.317</td>
</tr>
<tr>
<td>Treatment recommended by doctor (No/yes)</td>
<td>1.260712 (95%)</td>
<td>0.128</td>
</tr>
<tr>
<td>Number of family members having malaria (No/yes)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 4.3 Multivariate table of microscopy positive samples

<table>
<thead>
<tr>
<th>Variables</th>
<th>Odds ratio (confidence interval)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>General health (compared with poor health)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weak</td>
<td>0.8698489 (95%)</td>
<td>0.515</td>
</tr>
<tr>
<td>Satisfactory</td>
<td>0.2413133 (95%)</td>
<td>0.000</td>
</tr>
<tr>
<td>Sleeping habit (compared to outside)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inside</td>
<td>0.6898804 (95%)</td>
<td>0.022</td>
</tr>
<tr>
<td>Health care facility in living area (No/yes)</td>
<td>0.6086407 (95%)</td>
<td>0.003</td>
</tr>
</tbody>
</table>

4.5.3 Univariate risk factors analysis for RDT positive samples

1618 individuals including 264 positive cases and 1354 controls, were matched by gender and age categories to derive 264 matched pairs in Stata Statistical Software: Release 13 (StataCorp LP, 2013). Using this case-control data, risk factor assessment for RDT was performed through univariable and step-wise multivariable conditional logistic regression in Stata v13.

Univariate risk factors analysis for RDT detected samples showed general health condition (satisfactory OR=0.58, P=0.04, 95%CI and weak OR=0.71, p=0.00, 95%CI) have significantly protective effect for malaria infection.
Table 4.4 Univariate risk factors analysis of RDT positive samples

<table>
<thead>
<tr>
<th>Variable</th>
<th>Odd ratio (confidence interval)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age group (compared to &lt;5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;5</td>
<td>0.9866667 (95%)</td>
<td>0.951</td>
</tr>
<tr>
<td>&gt;20</td>
<td>0.9758242 (95%)</td>
<td>0.912</td>
</tr>
<tr>
<td>Sex (female compared to male)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Literacy (compared to illiterate)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary &amp; above</td>
<td>1.294421 (95%)</td>
<td>0.536</td>
</tr>
<tr>
<td>Secondary &amp; above</td>
<td>1.017045 (95%)</td>
<td>0.931</td>
</tr>
<tr>
<td>Duration of previous malaria illness (compared to no illness)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;15</td>
<td>0.8990181 (95%)</td>
<td>0.554</td>
</tr>
<tr>
<td>&gt;15</td>
<td>0.4253687 (95%)</td>
<td>0.073</td>
</tr>
<tr>
<td>Previous illness (yes/No)</td>
<td>0.8548892 (95%)</td>
<td>0.376</td>
</tr>
<tr>
<td>Recurrent malaria (No/yes)</td>
<td>1.391304 (95%)</td>
<td>0.485</td>
</tr>
<tr>
<td>Fever history</td>
<td></td>
<td></td>
</tr>
<tr>
<td>General health (compared with poor health)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weak</td>
<td>0.5815387 (95%)</td>
<td>0.048</td>
</tr>
<tr>
<td>Satisfactory</td>
<td>0.175814 (95%)</td>
<td>0</td>
</tr>
<tr>
<td>Domestic animals (No/yes)</td>
<td>1.276169 (95%)</td>
<td>0.163</td>
</tr>
<tr>
<td>Screening against malaria (No/yes)</td>
<td>1.043564 (95%)</td>
<td>0.836</td>
</tr>
<tr>
<td>House type (compared with Mud)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concrete</td>
<td>0.9699454 (95%)</td>
<td>0.861</td>
</tr>
<tr>
<td>Tent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water reservoir in vicinity (No/yes)</td>
<td>1.176 (95%)</td>
<td>0.688</td>
</tr>
<tr>
<td>Sleeping habit (compared to outside)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inside</td>
<td>0.7123756 (95%)</td>
<td>0.05</td>
</tr>
<tr>
<td>Blood group (compared to A blood group)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>0.4242424 (95%)</td>
<td>0.231</td>
</tr>
<tr>
<td>AB</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>O</td>
<td>3.888889 (95%)</td>
<td>0.26</td>
</tr>
<tr>
<td>Health care facility in living area (No/yes)</td>
<td>0.9466738 (95%)</td>
<td>0.774</td>
</tr>
<tr>
<td>Anti-malarial drugs intake (No/yes)</td>
<td>1.200354 (95%)</td>
<td>0.296</td>
</tr>
<tr>
<td>Socio economic status (compared with lower class)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lower middle</td>
<td>0.7501209 (95%)</td>
<td>0.187</td>
</tr>
<tr>
<td>Upper middle</td>
<td>0.7168911 (95%)</td>
<td>0.143</td>
</tr>
<tr>
<td>Upper class</td>
<td>1.088778 (95%)</td>
<td>0.817</td>
</tr>
<tr>
<td>Symptomatic fever (No/yes)</td>
<td>1.095278 (95%)</td>
<td>0.601</td>
</tr>
<tr>
<td>Chill Fever</td>
<td>1.197183 (95%)</td>
<td>0.428</td>
</tr>
<tr>
<td>Headache (No/yes)</td>
<td>1.18107 (95%)</td>
<td>0.618</td>
</tr>
<tr>
<td>Body ache (No/yes)</td>
<td>0.7584077 (95%)</td>
<td>0.138</td>
</tr>
<tr>
<td>Diarrhea (No/yes)</td>
<td>4.4046154 (95%)</td>
<td>0.213</td>
</tr>
<tr>
<td>Vomiting (No/yes)</td>
<td>0.8396761 (95%)</td>
<td>0.609</td>
</tr>
<tr>
<td>Anemic (No/yes)</td>
<td>2.007634 (95%)</td>
<td>0.57</td>
</tr>
<tr>
<td>Respiratory tract infection (No/yes)</td>
<td>0.6177606 (95%)</td>
<td>0.404</td>
</tr>
<tr>
<td>Treatment recommended by doctor (No/yes)</td>
<td>1.297297 (95%)</td>
<td>0.137</td>
</tr>
<tr>
<td>Number of family members having malaria (No/yes)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4.5.4 Risk factors analysis for PCR positive samples

Among 311 individuals including 95 positive cases and 216 controls, were matched by gender and age categories to derive 95 matched pairs in Stata Statistical Software: Release 13 (StataCorp LP, 2013). Using this case-control data, risk factor assessment for PCR was performed through univariable and step-wise multivariable conditional logistic regression in Stata v13.

Univariate risk factors analysis for PCR showed significant association between general health condition of individual (satisfactory OR=0.5, P=0.008, 95%CI and weak OR=0.33, P=0.04, 95%CI) and decrease in malarial infection risk.
### Table 4.5 Univariate risk factors analysis for PCR positive samples

<table>
<thead>
<tr>
<th>Variable</th>
<th>Odd ratio (confidence interval)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age group (compared to &lt;5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt; 5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>&gt; 20</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Sex (female compared to male)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Literacy (compared to illiterate)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary &amp; above</td>
<td>1.894345</td>
<td>0.12</td>
</tr>
<tr>
<td>Secondary &amp; above</td>
<td>1.515476</td>
<td>0.286</td>
</tr>
<tr>
<td>Previous illness (Yes/No)</td>
<td>1.241026</td>
<td>0.463</td>
</tr>
<tr>
<td>Duration of previous malaria illness (compared to no illness)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 15</td>
<td>1.32</td>
<td>0.356</td>
</tr>
<tr>
<td>&gt; 15</td>
<td>0.55</td>
<td>0.501</td>
</tr>
<tr>
<td>Recurrent malaria (No/yes)</td>
<td>1.348148</td>
<td>0.701</td>
</tr>
<tr>
<td>Fever history</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>General health (compared with poor health)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weak</td>
<td>0.5</td>
<td>0.038</td>
</tr>
<tr>
<td>Satisfactory</td>
<td>0.336</td>
<td>0.008</td>
</tr>
<tr>
<td>Domestic animals (No/yes)</td>
<td>1.102901</td>
<td>0.754</td>
</tr>
<tr>
<td>Screening against malaria (No/yes)</td>
<td>1.447619</td>
<td>0.295</td>
</tr>
<tr>
<td>House type (compared with Mud)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concrete</td>
<td>0.542005</td>
<td>0.054</td>
</tr>
<tr>
<td>Tent</td>
<td>0.569801</td>
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</tr>
<tr>
<td>Water reservoir in vicinity (No/yes)</td>
<td>0.489923</td>
<td>0.125</td>
</tr>
<tr>
<td>Sleeping habit (compared to outside)</td>
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<td></td>
</tr>
<tr>
<td>Inside</td>
<td>1.101075</td>
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</tr>
<tr>
<td>Blood group (compared to A blood group)</td>
<td></td>
<td></td>
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<td>-</td>
</tr>
<tr>
<td>AB</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Health care facility in living area (No/yes)</td>
<td>0.649123</td>
<td>0.143</td>
</tr>
<tr>
<td>Anti-malarial drugs intake (No/yes)</td>
<td>0.894085</td>
<td>0.738</td>
</tr>
<tr>
<td>Socio economic status (compared with lower class)</td>
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</tr>
<tr>
<td>Lower middle</td>
<td>1.244444</td>
<td>0.533</td>
</tr>
<tr>
<td>Upper middle</td>
<td>1.46087</td>
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</tr>
<tr>
<td>Upper class</td>
<td>1.217391</td>
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</tr>
<tr>
<td>Symptomatic fever (No/yes)</td>
<td>1.349876</td>
<td>0.344</td>
</tr>
<tr>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Chill fever (No/yes)</td>
<td>1.516484</td>
<td>0.653</td>
</tr>
<tr>
<td>Body ache (No/yes)</td>
<td>0.586813</td>
<td>0.475</td>
</tr>
<tr>
<td>Diarrhea (No/yes)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vomiting (No/yes)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Anemic (No/yes)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Respiratory tract infection (No/yes)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Treatment recommended by doctor (No/yes)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Number of family members having malaria (No/yes)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4.5.5 Univariate risk factors analysis for all positive cases

In univariate logistic regression model for cases detected by three diagnostic means (microscopy, PCR and RDT), the exposure variables which were statistically significant for malaria outcome included literacy level (OR=1.97 P=0.01, 95% CI), animals domesticated within houses (OR=1.35, P=0.027, 95%CI) and treatment recommendation by doctor (OR=1.84, P=0.00, 95%CI).

Table 4.6 All positive cases risk factors analysis

<table>
<thead>
<tr>
<th>Variable</th>
<th>Odd ratio (confidence interval)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age group (compared to &lt;5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>&gt;20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex (female compared to male)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Literacy (compared to illiterate)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary &amp; above</td>
<td>1.973333</td>
<td>0.01</td>
</tr>
<tr>
<td>Secondary &amp; above</td>
<td>1.062534</td>
<td>0.705</td>
</tr>
<tr>
<td>Duration of previous malaria illness (compared to no illness)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;15 months</td>
<td>1.042077</td>
<td>0.786</td>
</tr>
<tr>
<td>&gt;15 months</td>
<td>0.497462</td>
<td>0.068</td>
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<td>Previous Illness (No/yes)</td>
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<td>0.945</td>
</tr>
<tr>
<td>Recurrent malaria (No/yes)</td>
<td>1.401459</td>
<td>0.362</td>
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<tr>
<td>Fever history</td>
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<td></td>
</tr>
<tr>
<td>General health (compared with poor health)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weak</td>
<td>0.51024</td>
<td>0.001</td>
</tr>
<tr>
<td>Satisfactory</td>
<td>0.156651</td>
<td>0.00</td>
</tr>
<tr>
<td>Domestic animals (No/yes)</td>
<td>1.356831</td>
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</tr>
<tr>
<td>Screening against malaria (No/yes)</td>
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<tr>
<td>House type (compared with Mud)</td>
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<td></td>
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<tr>
<td>Concrete</td>
<td>0.857802</td>
<td>0.27</td>
</tr>
<tr>
<td>Tent</td>
<td>2.17734</td>
<td>0.093</td>
</tr>
<tr>
<td>Water reservoir in vicinity (No/yes)</td>
<td>1.091667</td>
<td>0.717</td>
</tr>
<tr>
<td>Sleeping habit (compared to outside)</td>
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<td></td>
</tr>
<tr>
<td>Inside</td>
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</tr>
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<td>Blood group (compared to A blood group)</td>
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</tr>
<tr>
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<td>0.275</td>
</tr>
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<td>1</td>
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<tr>
<td>O</td>
<td>2.4</td>
<td>0.354</td>
</tr>
<tr>
<td>Health care facility in living area (No/yes)</td>
<td>1.021853</td>
<td>0.883</td>
</tr>
<tr>
<td>Anti-malarial drugs intake (No/yes)</td>
<td>0.884773</td>
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</tr>
<tr>
<td>Socio economic status (compared with lower class)</td>
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<td></td>
</tr>
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<td>Lower middle</td>
<td>1.025686</td>
<td>0.881</td>
</tr>
<tr>
<td>------------------------</td>
<td>----------</td>
<td>-------</td>
</tr>
<tr>
<td>Upper middle</td>
<td>0.762584</td>
<td>0.129</td>
</tr>
<tr>
<td>Upper class</td>
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</tr>
<tr>
<td>Symptomatic fever (No/yes)</td>
<td>0.927839</td>
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</tr>
<tr>
<td>Chill fever (No/Yes)</td>
<td>1.041935</td>
<td>0.893</td>
</tr>
<tr>
<td>Headache (No/yes)</td>
<td>0.873514</td>
<td>0.653</td>
</tr>
<tr>
<td>Body ache (No/yes)</td>
<td>0.541876</td>
<td>0</td>
</tr>
<tr>
<td>Diarrhea (No/yes)</td>
<td>1.33647</td>
<td>0.705</td>
</tr>
<tr>
<td>Vomiting (No/yes)</td>
<td>0.639193</td>
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</tr>
<tr>
<td>Anemic (No/yes)</td>
<td>1.252948</td>
<td>0.738</td>
</tr>
<tr>
<td>Respiratory tract infection (No/yes)</td>
<td>0.539007</td>
<td>0.228</td>
</tr>
<tr>
<td>Treatment recommended by doctor (No/yes)</td>
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<td>0</td>
</tr>
<tr>
<td>Number of family members having malaria (No/yes)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4.5.6 Multivariate analysis for all positive cases (through all techniques)

Multivariate analysis of the samples detected through all techniques showed Literacy level (OR=1.98, \( P=0.017 \), 95%CI), treatment recommended by doctor (OR=1.47, \( P=0.012 \), 95%CI) are the significant risk factors for malaria infection. While Apparent health status (weak OR=0.56, \( P=0.005 \), 95%CI and satisfactory OR=0.17, \( P=0.00 \), 95%CI) and Sleeping inside the room (OR=0.71, \( P=0.028 \), 95%CI), body ache (OR=0.66, \( P=0.019 \), 95%CI) are the protective factors which shows statistical significance in preventing malaria.

Table 4.7 Multivariate analysis of significant factors

<table>
<thead>
<tr>
<th>Variables</th>
<th>Odds ratio (confidence interval)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Literacy (compared to illiterate)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary &amp; above</td>
<td>1.981171</td>
<td>0.017</td>
</tr>
<tr>
<td>Secondary &amp; above</td>
<td>1.251425</td>
<td>0.201</td>
</tr>
<tr>
<td>General health (compared with poor health)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weak</td>
<td>0.5601284</td>
<td>0.005</td>
</tr>
<tr>
<td>Satisfactory</td>
<td>0.1766944</td>
<td>0.000</td>
</tr>
<tr>
<td>Sleeping habit (compared to outside)</td>
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<td></td>
</tr>
<tr>
<td>Inside</td>
<td>0.7152879</td>
<td>0.028</td>
</tr>
<tr>
<td>Body ache (No/yes)</td>
<td>0.6640837</td>
<td>0.019</td>
</tr>
<tr>
<td>Treatment recommended by doctor (No/yes)</td>
<td>1.475297</td>
<td>0.012</td>
</tr>
</tbody>
</table>
4.6 Discussion

The study was conducted to find the relationship between malaria prevalence and environmental and socioeconomic variables. For this purpose, multilevel analysis of individual and household level factors was used to find their significance with risk of malaria.

Domestic and individual level malaria risk factors were reported in many studies, included access to health facility, house type, closeness to the vector breeding sites, vector abundance, socio economic status, occupation, gender, travel history, migration, frequent change of residence, presence of domestic animals, use of bed nets and other protective measures etc. (Kreuels et al., 2008, Ghebreysus et al., 2000, Staedke et al., 2003, Vittor et al., 2006, Tilaye and Deressa, 2007, Somi et al., 2007, Siri et al., 2010, Bogh et al., 2001). Ethnicity is also recognized as an independent risk factor for malaria (Hustache et al., 2007). The existence of mosquito breeding sites also determined by the variations in the geography of land parts (Balls et al., 2004). The risk to malaria infection is also varies with the distance to the mosquito breeding sites (Trape et al., 1992, Gamage-Mendis et al., 1991, Gunawardena et al., 1998). The interaction of these factors with malaria prevalence is variable in different geographical settings which needs to be investigated in each locality along with correlated climatic factors. The impact of these parameters differs with fluctuations of seasonal transmission of the localities (Carter et al., 2000, Antonio-Nkondjio et al., 2005, Cano et al., 2006).

Researchers have since seen that age-specific human attack rates peaked in adult males, suggesting that occupation may be an important risk factor in malaria transmission (Roper et al., 2000). The same study found that agriculturists were 3.2 times as likely to have a malaria episode compared to individuals working in commerce. The travelling history is also considered as important risk factor for malaria (Chuquiyauri et al., 2012).
Keeping livestock indoors was seen as a risk factor for acquiring malaria as described previously (Khan et al., 2013; Bouma & Rowland, 1995). Cattle may attract mosquitoes, either by serving as bait or by creating mosquito breeding and resting sites near livestock pens (Tuyishimire, 2013; Mahande et al., 2007; Peterson et al., 2009; Siri et al., 2010). Cattle provide an easy source of blood to mosquitoes, which results into increased vector populations, a significant proportion of which is attracted to feed on individuals sleeping outdoors near animals (Wahid, 2013). It is most likely that the risk of mosquito bites could have been reduced due to zoo-prophylaxis (Kaburi et al., 2009) especially in situations where vector species predominantly display zoophagic foraging tendencies. However, the benefit of keeping livestock close to human dwellings has been refuted by many authors (Bogh et al., 2001; Ghebreyesus et al., 2000). In Pakistan, Anopheles culicifacies and A. stephensi are confirmed as malaria vectors (Jahan & Sarwar, 2013) and are chiefly Pakistan’s zoophilic (Naz et al., 2013). A. culicifacies is seen as the primary vector in rural areas while A. stephensi considered to an important vector for malaria transmission in urban areas (Carmichael, 1972; Reisen & Boreham, 1982). Blood meal analyses conducted by Reisen and Boreham (1982) in Punjab revealed just 1% of blood meals in Anopheles stephensi and 5% in An. culicifacies to be of human origin. Livestock are thought to be largely responsible for generating the high mosquito densities in the region, a strong positive correlation between cattle:man ratio and malaria incidence was reported in northern KP province of Pakistan (Bouma & Rowland, 1995).

Concrete houses appeared to reduce risk of malaria since they can decrease mosquito contact by limiting their entry and reducing their resting places (Ayele et al., 2012). Higher malaria incidence rate is commonly reported from poorly constructed houses (incomplete, mud, or palm walls and palm thatched roofs) compared to well-
constructed houses (bricks/stones) since poor housing likely leads to an increase in human-vector contact (Gamage-Mendis et al., 1991; Keiser et al., 2004; Ayele et al., 2012; Ghebreyesus et al., 2000; Gunawardena et al., 1998; Koram et al., 1995). On the contrary, several others suggest no such association between malaria incidence and housing quality (Peterson et al., 2009; Kimbi et al., 2013; Van Der Hoek et al., 1998; Wolff et al., 2001; Lenz, 1988; Snow et al., 1998).

The poorly constructed houses or mud built houses in rural settings contributes more in malaria infection by providing more resting places and entrance points into houses for mosquitoes in malaria prevalent areas, other studies also reported high prevalence of disease in rural areas (Kimbi et al., 2013, Chirebvu et al., 2014) where poorly constructed houses and keeping domesticated animal inside mud built houses can be one of the major reason by serving as bait or by creating mosquito breeding and resting sites near livestock pens, rural travel was strongly associated with adult malaria incidence while urban travel was not found to be associated with malaria risk (Peterson et al., 2009). Similarly, in Southeast Asian countries, living in poorly built houses (mud, palm walls, and palm thatched roofs) were associated with increased risk of malaria that was suggested to increase vector-human contact (Arasu, 1992, Oemijati, 1992). For example, a study conducted in Thailand demonstrated that poor housing conditions (mud, palm walls and palm thatched roofs) increased the risk of malaria 19-fold (Butraporn et al., 1986). Similarly, in India better housing conditions (bricks walls/cemented or iron sheet roof with no eaves) in town was associated with low risk of malaria prevalence than rural areas that were thought to limit access to vector mosquitoes (Dev et al., 2004).

Transmission in and around the house remains significant, as indicated by the statistical analyses which showed that the type of housing, unavailability of in-house toilets, and
the presence of uncovered water containers close to the houses are also significant predictors of malaria in Hadhramout. Thus, it will be important to improve the environment and economic status of the inhabitants (Bamaga et al., 2014).

Other studies also reported significant association between house type and malaria outcome (Woyessa et al., 2013, Coleman et al., 2010). People with poor housing quality are at great risk of malaria as it provides entrance to the mosquitoes inside houses. The houses construction pattern is reported as greater risk associated with malaria infection (Chanon et al., 2003, Schofield and White, 1984, Mendis et al., 1990, Konradsen et al., 2003).

Living in an upper middle class was protective against malaria as these households have the amenities to adopt better preventive measures compared to the poor and underprivileged (Koram et al., 1995; Guthmann et al., 2001; Ong'echa et al., 2006; El Samani et al., 1987; Banguero, 1984; Sintasath et al., 2005; Macintyre et al., 2002).

Most of the people of rural areas belongs to poor class which are the major victims as they are less privileged and economically downtrodden people, who often have dirty environments and most often have no means of acquiring better mosquito-bite preventive measures, and no access to modern treatment and found to be at greater risk of malaria disease because they are poorer and face higher transmission rates than their urban counterparts as Social and economic factors aggravate the contribution of climate related factors in influencing the malaria burden (Macintyre et al., 2002, Somi et al., 2007, IndexMundi., 2013, Robert et al., 2003, Henry et al., 2003). Socioeconomic factors may directly or indirectly affect malaria transmission (Temel, 2004; Brooker et al., 2004; Mcmichael et al., 2006) and failure to consider effects of socioeconomic elements might jeopardize the success of control programs (Al-Taïar et al., 2009).
According to the World Health Organization sedentary communities living in malaria prone areas who cannot afford to move out of flood affected areas due to low economic status have an increased chance of acquiring infection. This is particularly true for children, pregnant women, and older people, who form the high-risk groups (WHO, 2011a). Other studies also reported no significant associations (Luckner et al., 1998, Niringiye and Dougason, 2010) which contradicts the assertion that poverty undermines the coping mechanisms that could help poor people reduce their vulnerability to malaria (Yanda et al., 2006).

The low socio economic status (SES) was reported significant for increasing malaria risk in previous studies (Thang et al., 2008, Cook, 2010, Feachem et al., 2010, Ayele et al., 2012, Chirebvu et al., 2014). This may be variety of factors such as repellent affordability, poor hygiene, poor house construction which contribute to increased risk of malaria in lower income class as compare to higher status. It has also been reported that the wealthy and educated households often live in clean environments, and can afford better mosquito-bite preventive measures, and good medical attention when afflicted with malaria. The major victims are usually the poor, less privileged and economically downtrodden people, who often have dirty environments, and most often, have no means of acquiring better mosquito-bite preventive measures, and no access to modern treatment (Robert et al., 2003, Kimbi et al., 2013).

The risk of being infected with malaria was up to 2.5 times greater for people in poorly constructed houses, with thatched roofs and mud walls than for people in better-constructed houses (Charlwood et al., 2015, Obaldia, 2015, Gamage-Mendis et al., 1991, Gunawardena et al., 1998, Konradsen et al., 2003, Pinikahan, 1992, van der Hoek et al., 1998). Higher socio-economic level (according to equipment and education levels) was associated with a significantly lower risk of infection as the higher socio-
economic status makes easier the access to anti-parasite interventions, *i.e.* anti-malarial treatment, more than to anti-vector measures independent of bed net use (Baragatti et al., 2009).

Dale *et al.* found that low-middle income and lower education levels were significantly associated with malaria in Indonesia (Dale et al., 2005). A study in Thailand showed a longer residence duration (adjusted OR = 0.36, 95% CI) and the use of anti-malarial self-medication (adjusted OR = 0.08, 95% CI) were significantly associated with protection from severe malaria (Hustache et al., 2007). Koram *et al.* also found that children living in poor quality housing and crowded dwellings were infected with malaria more frequently than other children living in better housing conditions in peri-urban areas in Gambia (Koram et al., 1995). Ijumba *et al.* demonstrated that the disappearance of malaria in some areas of Europe was associated with economic development and there is enough historical evidence to support that economic development has a positive impact on health.

Commonly, individuals availing government health services belong to lower socioeconomic backgrounds generally with low education levels. These have been associated with poor knowledge regarding utilization of preventative methods and malaria treatment among them (Tuyishimire, 2013). Achieving higher education is known to be protective against malaria (Dike et al., 2006). In Bannu, acquiring only primary education by patients or guardian of patients seemed to increase the risk of malaria. There is a dire need to disseminate information for preventive and control strategies in the study area as 1588 individuals denied using any precautionary measures.

Better education also helps the individuals to explain their illness to health workers as compared to illiterate ones (Krause et al., 1998). High level of education was found
significantly protective against malaria and the possible reason could be that people who have high level of education are better able to protective themselves as most of them belongs to higher class. However, the high level of education is not necessarily related to appropriate knowledge as in the same study there were only 20% of the individuals which had gone through beyond primary level of education while 55% of the individuals had better knowledge of protection against mosquito’s bites (Guthmann et al., 2001). The similar finding was reported in Gambia where 10% of the mothers had formal education beyond primary level but they had fair knowledge about malaria (Koram et al., 1995). Delayed diagnosis and treatment seeking attitudes, conditioned by the perception of the disease, could explain the increased risk of malaria in people who have poor knowledge of malaria (Hla et al., 1998, Weber et al., 2003).

In third world countries, the higher illiteracy level is reported among females as compared to males having better educational opportunity, experience, and unrestricted mobility, leads to less knowledge of illness and treatment of malaria while literate women have significantly higher demand for the utilization of malaria treatment and preventative methods than their illiterate counter-parts (Lampietti et al., 1999).

Apparent health status of the individual was indicative of malaria possibly because many patients were referred by clinicians for malaria tests due to their anemic status expected in malaria infections (Ekvall et al., 2001; Sumbele et al., 2010; Kimbi et al., 2013; Tarimo, 2007). If left untreated (or inadequately treated), malaria may result in several weeks or months of poor health following repeated attacks of fever and anemia (UNICEF, 2000).

Sutcliffe et al. reported 10-20% of the positive individuals with fever at the time of testing while many of the positive individuals were asymptomatic which poses a significant challenge to malaria elimination as many control strategies rely on the
identification and treatment of symptomatic individuals seeking care at health centers (Sutcliffe et al., 2011, Moonen et al., 2010).

Prompt and accurate diagnosis of malaria is the key to effective disease management and therefore it is one of the main interventions of the global malaria control strategy. Identification of the species of malarial parasite is very important for its effective and curative treatment as resistance to chloroquine and other anti-malarial drugs has been reported previously (Rahim and Younas, 2003, Atif, 2009). For accurate diagnosis and treatment easy access to health care and antimalarial drugs, and greater willingness to take sanitary measures can play a vital role in reducing malaria burden (Robert et al., 2003, Klinkenberg et al., 2004). In study area, many of the patients lacking access to the public health services which leads to increasing incidence of malaria as many of them use self-medication which fails in curing malaria, the self-intake of antimalarial drugs leads to incorrect dosage consumption which cause the increase resistance of *Plasmodium* to routine dosage. The rapid increase of both anti-malarial drug resistance and insecticide resistance is a major problem which restrains the control of malaria. Therefore, the WHO introduced two powerful and broadly used intervention tools, i.e. insecticide-treated nets (ITN) and indoor residual spraying (IRS) to reduce the intensity of local malaria transmission. Natural disasters are considered to accelerate the spread of malaria infection. The improved access to health care facility can be one of the effective malaria control strategies (Reiter, 2001, Suh et al., 2004).

Frequent outdoor activities also increased probability of infection as mosquitoes are generally active between dusk and dawn (Dale et al., 2005). Risk of malaria transmission always exists where mosquito host seeking behavior coincides with place and times of human presence (Ndoen et al., 2011). In the study, herein, most of
individuals had evening activities while sleeping in open grounds was a common practice.

Sleeping habits especially clustering in sleeping rooms was established as a crucial risk factor. Clustering at household level appears to increase malaria risk probably due to increased release of human chemo-attractants (Torres-Estrada & Rodriguez, 2003; Danis-Lozano et al., 2007). The study area experiences long hours of power outage in summers that aggravates the biting rate. Sleeping of more than one member on a single bed reflects the lower economic condition of the family, while the increased amount of human released chemo-attractants can possibly increase the mosquito attraction inside the rooms and can increase the risk of malaria. Similarly sleeping of more individuals inside single room can also increase mosquito biting rate as human chemo-attractants increased (Torres-Estrada and Rodriguez, 2003, Danis-Lozano et al., 2007). In the urban center of study area most of the members sleeping in single room faced severe load shedding problem with no alternative which lead to high influx of mosquitoes inside the room and chemo-attractants may also contributes to increased biting rate. Infected houses members also increased the risk of spreading malaria.

In study area, most of the population belongs to lower socioeconomic class who can’t afford the good quality repellents and other protective measures. Illiteracy also contributes to ignorant behavior about protective measure. While locally made affordable repellents are of low quality which didn’t fulfill he need. In poorly constructed houses the sleeping and cooking is also performed in same room, smoke could act as repellent but not useful when meal is prepared outside room or when room windows are unglazed and cracks in door or in walls (Ghebreyesus et al., 2000). Study done in Kenya showed that sleeping in a room with sprayed insecticides reduce the risk of infection by 75% while using ITN can reduced the risk by 63% (Guyatt et al., 2002).
Dale et al. found that outdoor activities are a higher risk behavior as mosquitoes are most active between dusk and dawn (Dale et al., 2005). The highest risk of malaria transmission is where mosquito host seeking behavior coincides with places and times of human presence (Ndoen et al., 2011). In the study area, most of the individuals have evening activities while sleeping in open grounds at night is a common practice which may increase the risk of malaria.

Many of the previous studies reported positive relation between apparent symptoms and malaria outcome. Each attack of malaria may last several hours with several symptoms, often begins with shivering (body shaking), fever and profuse sweating. During an attack, the patient often complains of headache and pain in the back, joints and all over the body. Other symptoms included loss of appetite, vomiting and diarrhoea. While the child may present with symptoms of severe malaria such as loss of consciousness, drowsiness and/or convulsions, diarrhoea, dark urine and reduced urine output (anuria). While many of the anti-malarial drugs have side-effects including headache and body aches (UNICEF, 2000). The present study also showed significant relation of body aches and malaria. Malarial infection leads to ischemia due to sequestration of red blood cells, inflammation and oxidative stress, which in turn damages skeletal and cardiac muscles. Malaria induces a combination of inflammation and oxidative destruction in skeletal and cardiac muscles leading to the increased degradation of contractile proteins which results in compromised muscle function (Marrelli and Brotto, 2016).

Statistically significant relation was not found between headache and malaria outcome in this study but previously it is reported as important presentation of malaria before and during treatment. Headache profile in patients with malaria focusing on its mechanism. Headache is an important presentation in malaria, either cerebral type or
not. The cytokine is believed to be an important factor leading to headache in acute malaria. Some antimalarial drugs can cause headaches. In addition, headache is one of the symptoms of post malaria neurologic syndrome (Wiwanitkit, 2009).

According to the WHO guidelines, Mozambique is in the process of adopting a case management policy that recommends all cases of suspected malaria be confirmed by laboratory testing (Tiago et al., 2011). This policy serves at least two purposes: reduce unnecessary use of artemisinin-based combination therapy and exclude malaria among non-malaria febrile patients, allowing for other causes of fever to be investigated and treated. Overtreatment of malaria is not only an economic concern; it has been proposed that restricting antimalarial use to laboratory-confirmed cases will also delay the emergence and spread of resistance to artemisinin derivatives and their partner drugs (Malisa et al., 2010). Of even more concern is the issue of labeling febrile patients with malaria. Some studies have shown a reduction in the prevalence of malaria parasitaemia among febrile patients over the last decades (D'acremont et al., 2010; Waitumbi et al., 2010; Reyburn et al., 2004). The labeling of all febrile patients as having malaria can have severe consequences as the underlying disease would not be properly identified and treated (Reyburn et al., 2004). In our study, we observed that although many of the patients were recommended by clinicians for diagnosis, there were several patients that visited the facility on their own behalf. Many had a history of malaria with self-medication while others were prescribed anti-malarials without diagnosis. Many of the private practitioners and labs also prescribed antimalarial drugs without confirmatory malaria tests. The cases of improper intake of anti-malarial drugs were also observed during study as lack of awareness and careless attitude towards health is a common practice in the area. For prevention of all these ill practices, government should make
sure that without doctor’s recommendation and reliable diagnostic results the antimalarial drugs are not provided to individuals.

Previous malarial infections lead to rapid acquisition of protection by developing immunity against malaria which is reported in previous studies (Gupta et al., 1999, Phillips, 2001). Patients having no previous history of malaria showed more severe disease condition compared to those having 1-3 episodes of clinical malaria. Little exposure to malaria can lead to acquiring immunity against disease which can protect against severe disease while in children it leads to rapid acquisition of protection against infection (Gupta et al., 1999, Mackinnon et al., 2005).

In this study, we also ask about previous history of malarial infection and many of individuals have previous infection history but in contrast to other studies significant association between malarial infection and previous history of malaria was not found in the present study.

The previous clinical infection play an important role in secondary infection as the Vivax hypnozoites survive in liver cells for long time period (Garnham, 1967, Garnham, 1987) while the reactivation of these parasites leads to relapse of infection in individual (Hankey et al., 1953) and the role of these relapses in malaria outbreaks are documented (Feighner et al., 1998, Chadee et al., 1992). In Mexico, the collection of new and secondary cases together results in 80% of malaria cases in endemic regions. The family member having clinical episode of malaria are the greater risk for other healthy individuals of the house as he can harbor the parasite for several years and can also present symptomatic and asymptomatic relapses (Danis-Lozano et al., 2007).

Age is another factor reported as significant in many of previous studies (Dondorp et al., 2008, Ferede et al., 2013) while in this study age is not found statistically significant. Children in the youngest age (≤ 6 years) had significantly high malaria parasite
prevalence when compared to the older ones (Bodker et al., 2003, Brooker et al., 2004, Peterson et al., 2009). The acquired immunity is both exposure- and age-dependent, and the older children are likely to have developed some degree of immunity, because of repeated infections (Bodker et al., 2006) conducted in Butajira area of Ethiopia reported highly significant statistical relation between low age and malaria incidence (Woyessa et al., 2013). The possible reason could be the illiteracy of the guardians towards protective measures and overlapping of different activities of children and Anopheles biting behavior (Peterson et al., 2009).

Surprisingly gender as significant factor was not found in this study while previous studies reported significant association between male gender and risk of malaria as males expose their bodies more often than females, especially when the weather is hot, thus, increasing their chances of being bitten by mosquitoes, males engage in activities which make them more prone to infective mosquito bites as compared to females’ counterparts which are mostly at home and protected from such infective bites (Kimbi et al., 2013, Ferede et al., 2013, Alemu et al., 2012, Mandel et al., 1984). While in contrast other studies reported female at greater risk of malaria because of their schedule than their male counter parts. Women are often at work before dawn and continue late into the night when the mosquitoes are active (Tanner and Vlassoff, 1998, Ghebreyesus et al., 2000). They wake up during early mornings to prepare food and stay up late to clean and to take care of other household chores in the evening. In addition, women fetch water from rivers, dams, and water reservoirs, in most cases early in the morning. Women also wash family clothes and household items by going to these rivers and water reservoirs. These water sources harbor a large population of mosquitoes increasing women’s chances of getting malaria. In most cases men usually do not involve in household chores.
The use of protective measures such as topical repellents, domestic insecticides, smoke, bed nets etc. are reported significant in many studies (Stefani et al., 2011, Pajot et al., 1977) but statistically significant association could not be confirmed in this study. The possible reason can be the improper usage and quality of the protective substances and measures. Poor knowledge about disease and its prevention could be the greater risk for increasing malaria incidence (Valle and Torres, 1965). Intensity of malaria transmission, poor housing condition and poor protection against malaria can be the possible causes of the increase malaria risk (Danis-Lozano et al., 2007).

The movement of infected people from areas where malaria is endemic to areas with low rates or malaria-free can lead to resurgence of disease (Dale et al., 2005). Conversely, non-immune or low immune people are prone to be infected by malaria if they travel or move to malaria endemic regions (Reiter, 2001). Huge population movement which increases the risk of malaria transmission (Zhou et al., 2005). Many internally displaced population (IDPs) was settled in Bannu in the study duration which was an important factor for contributing increased malaria incidence in Bannu. But statistically significance between migrants and malaria outcome in the region was not found.

4.6.1 Strategies

For controlling malaria it is necessary to adopt new control strategies such as reducing mosquito abundance by treating larval breeding sites. Controlling the relapses of Vivax by repeated use of chloroquine and primaquine is another way for reducing burden. Other strategies including involvement of community participation and periodic clearing of vegetation around houses, raising education level to improve knowledge about malaria diagnosis and seeking treatment, use of protective measure while travelling, increase use of treated bed nets are useful (Chanon et al., 2003, Rozendaal
et al., 1989, Burkot et al., 1990, D'Alessandro et al., 1995, Lindblade et al., 2004, Charlwood et al., 2003, Rhee et al., 2005).
Chapter 5  Population genetics of *Plasmodium vivax*

5.1 Introduction

Current research is mainly focused on *Vivax* and *falciparum* malaria and little efforts are contributed towards other human malaria species. Beside mono-infection by these species, mixed infection within one individual can occur which play an important role in progression and outcomes of disease (Richie, 1988, Bruce et al., 2000).

The species identification is performed through microscopy but its sensitivity and specificity is related with several factors. Species involved in multiple infections can be confirmed through molecular techniques while there several genotypes or strains of same species can also be confirmed through molecular methods (Luxemburger et al., 1997, May et al., 2000). Naturally significant number of multiple infections are caused by different genotypes of same species within the single host which is referred as multiplicity of infection (MOI). In case of falciparum, MOI is related with several factors such as age of the host, clinical severity and transmission intensity. Determination of within-host diversity may be a useful marker to assess the impact of interventions (Smith et al., 1993, Mayengue et al., 2009, Juliano et al., 2010).

*P. vivax* is widely spread human malaria species and half of the world population is estimated to be at risk of *vivax* malaria (Price et al., 2007, Price et al., 2009, Guerra et al., 2006). In previous studies, it was reported that genetic variability also exists among members of multi-genes families of *P. vivax* (Neafsey et al., 2012). Mutation can help the parasite to evade the human immune response and to tolerate the drugs or stabilize the structure and function of proteins, proved by analyzing the field isolates (Wang et al., 2014, Chan et al., 2015). Novel sequencing techniques can help to find genetic diversity of *P. vivax* which is significantly important to monitor genes involved in drugs
susceptibility and to identify potential vaccine candidates (Amambua-Ngwa et al., 2012, Mobegi et al., 2014, Conway, 2015, Ariey et al., 2014).

Due to increased prevalence of *P. vivax* there is stronger need for new drug development, several genome projects are underway for this purpose but still there is low published data of vivax genome. Most of the sequencing projects are focused on the regional characteristics of *P. vivax* isolates (Winter et al., 2015), (Dharia et al., 2010).

The origin and evolutionary history of human malaria parasites, *P. falciparum* and *P. vivax*, the mitochondrial genome of Whole mt genome analyses of most common human malaria parasites, *Plasmodium falciparum* and *Plasmodium vivax*, have provided evidence of the origin and evolutionary histories of these species (Joy et al., 2003, Mu et al., 2005). Mitochondrial genome (partial or complete sequence) is useful marker for population studies in wide range of organisms (Avise and Walker, 1998, Jorde et al., 1998, Saccone, 1994). In *Plasmodium* genus, mitochondrial genome is specifically convenient for population studies as it is only 6kb in length and include only three genes i.e.; cox3, cox1 and cytb (Wilson and Williamson, 1997). Mitochondrial genome was used to find out the age of most recent common ancestor (MRCA) of *falciparum* (Hughes and Verra, 2002). As compared to *falciparum* there is less knowledge regarding population genetics of *P. vivax*. According to recent SNP analysis of nuclear genome, the polymorphism in *vivax* is similar or slightly higher than *falciparum* (Joy et al., 2003). The evolutionary studies of *falciparum* were aided by available isolates of *P. reichenowi* (chimpanzee malaria parasite), so it can be assumed that MRCA of *falciparum* and *reichenowi* occurred around the time that human and chimpanzee lineages diverged (5-7 million years ago). Thus *P. reichenowi* provides a calibration point for estimating divergence time within *falciparum*. Such a calibration
Mitochondria are ubiquitous organelles in all eukaryotes that are essential for a range of cellular processes and cellular signaling. Nearly all mitochondria have their own DNA or mitochondrial (mt) genome, which varies considerably in size, structure and organization. The phylum Apicomplexa includes a variety of unicellular eukaryotes, some of which are parasites of clinical or economic importance. Recent studies have demonstrated that apicomplexan mt genomes, which include the smallest 6 kb genome of the malaria parasites, exhibit remarkably diverse structures (Hikosaka et al., 2013). The genomes of *Plasmodium* mitochondria have only three protein-coding genes (cytochrome c oxidase subunits I and III: cox1 and cox3 and cytochrome b: cyt b) and highly fragmented rRNA genes have been identified. *Plasmodium* has the smallest mt genome in the form of a circular and/or tandemly repeated linear element of 6 kb (Preiser et al., 1996). Copy numbers for this element are approximately 20-fold and 150-fold of the nuclear genomes in *P. falciparum* (Preiser et al., 1996) and *P. yoelii* (Vaidya and Arasu, 1987), respectively. The 6-kb element encodes only three mt protein-coding genes (cox1, cox3 and cob) in addition to the large subunit (LSU) and small subunit (SSU) ribosomal RNA (rRNA) genes (Feagin et al., 1997). The two rRNA genes are highly fragmented with 20 rRNA pieces having been identified (Feagin et al., 1997, Hikosaka et al., 2011). Very recently, eight additional fragmented rRNA candidates have been identified in the *P. falciparum* mt genome (Feagin et al., 2012). Curiously, no transfer RNA genes have been identified.

There is little literature about *P. vivax* genome sequencing due to the reason it is not amenable to culture *in vitro*. Genetic diversity of *P. vivax* is reported from Pakistan is poorly studied and diversity based on whole genome sequences has not been reported.
to best of our knowledge. The mitochondrial genome sequence of prevalent malarial species in Pakistan is yet to be analyzed and compared to global mt genome sequences. To complete the evolutionary history of globally prevalent malarial parasite, the Pakistani genome data is necessary to be included.

There is high diversity of both *P. vivax* and *P. falciparum* populations in Bannu district. High prevalence of mixed specie and mixed-clone infections in *P. falciparum* and *P. vivax* in Bannu may be attributed to introduction of parasite (through frequent human travel) and high transmission rate. Bannu is basin like, and is intersected by two major rivers whose banks and associated irrigation activities provide ideal breeding grounds for malaria vectors. Secondly, besides being a regional commercial hub for Central Asia, the area has a high influx of Afghan refugees who may also facilitate the introduction of parasite clones from Afghanistan (Yang et al., 2006, Anderson et al., 2000, Vafa et al., 2008). Such heterogeneity might thus cause differences in parasite virulence, transmissibility and responses to chemotherapy, with important implications for malaria control measures in this populous region (Khatoon et al., 2010).

In this study, clinical isolates were obtained from District Bannu, Khyber Pakhtunkhwa were typed for mitochondrial genome to elucidate the status of *P. vivax* epidemiology in south region of Khyber Pakhtunkhwa province of Pakistan in relation to the global samples.

### 5.2 Results

Participants were recruited from the three labs of Bannu. The hospital's catchment area covers the entire district. Inclusion criterion was suspected malaria patients visiting the labs, irrespective of age or sex. PCR results of these samples showed that 25%, 5.14% and 69.4% of the samples (n=311) had *P. vivax*, *P. falciparum* and parasite-free respectively, while 14.46% were misdiagnosed by microscopy method. The same
sample were utilized to study the population structure of the *P. vivax* in the Bannu district of Pakistan. The positive samples were sent to LSHTM (London school of Hygiene and tropical medicine) for mitochondrial genome sequencing. Eleven different primers were designed and used for sequencing of mitochondrial DNA. Complete mitochondrial genome sequence was not obtained as clinical isolated were used in this study. In addition, data from Preston et al. et al. provided dataset for other geographical areas. Unfortunately, due to missing nucleotides sequences the number of haplotypes in the study area could not reported. Three mitochondrial genes i.e., cox1, cox3 and cytb were analyzed and mutation on different sites were recorded (Preston et al., 2014). Two hundred and thirty-one isolates from 18 countries were included in SNP analysis. Along with Pakistani samples all other countries SNP data was provided by LSHTM laboratory. Samples related to one country was considered as single population thus 18 population were included in this study for different analysis. These 18 populations were further categorized into five geographical regions described by WHO (WHO, 2017).

**Table 5.1 Data distribution into WHO malaria regions**

<table>
<thead>
<tr>
<th>Cluster name</th>
<th>Number of isolates</th>
<th>Counties included (number of samples)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Americas</td>
<td>20</td>
<td>Colombia (3), Peru (10), Brazil (4), Panama (1), Mexico (2)</td>
</tr>
<tr>
<td>Western Pacific</td>
<td>92</td>
<td>China (1), Cambodia (57), Malaysia (6), Vietnam (12), PNG</td>
</tr>
<tr>
<td>South East Asia</td>
<td>94</td>
<td>India (1), Thailand (86), Myanmar (1), Indonesia (5), Sri</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lanka (1)</td>
</tr>
<tr>
<td>Eastern Mediterranean</td>
<td>21</td>
<td>Pakistan (21)</td>
</tr>
<tr>
<td>African Region</td>
<td>1</td>
<td>Madagascar (1)</td>
</tr>
</tbody>
</table>
5.2.1 Basic properties of the analysed data

Table 5.2 shown the details of all populations. Highest number of samples were from Thailand (88) and Cambodia (57). The total length of alignment utilized is ~6Kb. The Salvador 1 strain was used as a reference. The missing data is represented by alphabet “N”.

Highest number of polymorphic loci was detected in PNG (78) among 208 usable loci, followed by Thailand (28) with 201 usable loci. The highest number of usable loci was found in Pakistan i.e. 224. The overall mean of polymorphic sites was 9.6.

Table 5.2 General properties of populations

<table>
<thead>
<tr>
<th></th>
<th>No. of gene copies</th>
<th>No. of usable Loci</th>
<th>No. of polymorphic loci</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salvador</td>
<td>1</td>
<td>180</td>
<td>0</td>
</tr>
<tr>
<td>Brazil</td>
<td>4</td>
<td>182</td>
<td>2</td>
</tr>
<tr>
<td>Cambodia</td>
<td>57</td>
<td>203</td>
<td>23</td>
</tr>
<tr>
<td>China</td>
<td>1</td>
<td>181</td>
<td>0</td>
</tr>
<tr>
<td>Colombia</td>
<td>3</td>
<td>181</td>
<td>0</td>
</tr>
<tr>
<td>India</td>
<td>1</td>
<td>181</td>
<td>0</td>
</tr>
<tr>
<td>Indonesia</td>
<td>5</td>
<td>188</td>
<td>7</td>
</tr>
<tr>
<td>Madagascar</td>
<td>1</td>
<td>181</td>
<td>0</td>
</tr>
<tr>
<td>Malaysia</td>
<td>6</td>
<td>181</td>
<td>8</td>
</tr>
<tr>
<td>Mexico</td>
<td>2</td>
<td>181</td>
<td>0</td>
</tr>
<tr>
<td>Myanmar</td>
<td>1</td>
<td>181</td>
<td>0</td>
</tr>
<tr>
<td>Pakistan</td>
<td>21</td>
<td>224</td>
<td>11</td>
</tr>
<tr>
<td>Panama</td>
<td>1</td>
<td>181</td>
<td>0</td>
</tr>
<tr>
<td>Peru</td>
<td>10</td>
<td>181</td>
<td>1</td>
</tr>
<tr>
<td>PNG</td>
<td>16</td>
<td>208</td>
<td>78</td>
</tr>
<tr>
<td>Sri Lanka</td>
<td>1</td>
<td>181</td>
<td>0</td>
</tr>
<tr>
<td>Thailand</td>
<td>88</td>
<td>201</td>
<td>28</td>
</tr>
<tr>
<td>Vietnam</td>
<td>12</td>
<td>189</td>
<td>15</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td><strong>12.8</strong></td>
<td><strong>188.1</strong></td>
<td><strong>9.611</strong></td>
</tr>
<tr>
<td><strong>SD</strong></td>
<td><strong>23.1</strong></td>
<td><strong>12.6</strong></td>
<td><strong>19.1</strong></td>
</tr>
</tbody>
</table>

Table 5.3 shows the variables sites found in Pakistani sample alignment. The 32 variable sites (SNP) were reported from Pakistani samples including 28 private SNPs. Most of the SNPs are noted at non-coding region of the genome.
| Position | 240 | 667 | 668 | 676 | 704 | 958 | 1054 | 1270 | 1291 | 1292 | 1295 | 1950 | 1973 | 3301 | 3313 | 3476 | 3490 | 3516 | 3522 | 3700 | 3778 | 3863 | 3982 | 4152 | 4435 | 4511 | 5357 | 5519 | 5911 | 5931 |
|----------|-----|-----|-----|-----|-----|-----|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|
| Ref allele | A   | G   | A   | A   | A   | G   | G   | T   | A   | C   | A   | T   | T   | G   | C   | A   | A   | G   | G   | G   | G   | G   | G   | A   | T   | G   | T   | C   | A   | A   |
| PAK1     | A   | G   | A   | A   | A   | G   | G   | T   | A   | C   | A   | T   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   |
| PAK5     | N   | T   | T   | T   | A   | A   | G   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   |
| PAK17    | N   | G   | A   | A   | A   | G   | A   | T   | A   | C   | A   | T   | T   | G   | C   | A   | -   | G   | G   | G   | G   | G   | G   | G   | N   | N   | N   | T   | C   | T   | A   |
| PAK19    | A   | G   | A   | A   | A   | G   | G   | T   | -   | -   | A   | G   | T   | G   | C   | A   | A   | A   | A   | A   | A   | A   | A   | A   | A   | A   | T   | C   | T   | C   | T   |
| PAK22    | A   | G   | A   | A   | A   | A   | G   | G   | T   | A   | C   | A   | T   | T   | G   | C   | A   | -   | A   | G   | A   | A   | A   | A   | A   | A   | A   | A   | T   | C   | T   |
| PAK23    | N   | G   | A   | A   | A   | A   | G   | G   | T   | A   | C   | A   | T   | T   | G   | T   | C   | A   | G   | G   | G   | G   | G   | G   | A   | T   | C   | T   | C   | T   |
| PAK25    | A   | N   | N   | N   | N   | N   | N   | T   | A   | C   | A   | T   | T   | G   | C   | A   | A   | G   | G   | G   | G   | G   | G   | G   | A   | A   | C   | G   | C   | C   |
| PAK26    | A   | N   | G   | A   | A   | A   | G   | N   | N   | N   | N   | N   | N   | N   | N   | C   | A   | -   | A   | G   | G   | G   | G   | G   | G   | A   | T   | C   | T   | C   | T   |
| PAK27    | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | G   | G   | N   | N   | N   | N   | N   | N   | N   | N   | N   |
| PAK28    | A   | G   | A   | A   | A   | A   | G   | G   | T   | A   | C   | A   | T   | T   | T   | N   | N   | N   | N   | N   | N   | N   | N   | N   | G   | A   | T   | C   | N   | N   | N   |
| PAK29    | A   | N   | N   | N   | N   | N   | N   | T   | A   | C   | A   | T   | T   | G   | C   | A   | A   | G   | G   | G   | G   | G   | G   | G   | A   | T   | C   | T   | C   | T   |
| PAK30    | A   | G   | A   | A   | A   | G   | G   | T   | A   | C   | A   | T   | T   | G   | A   | A   | G   | G   | G   | G   | G   | G   | G   | G   | A   | T   | C   | N   | N   | N   |
| PAK36    | N   | G   | A   | A   | A   | G   | N   | N   | N   | N   | N   | N   | T   | G   | C   | A   | A   | G   | G   | G   | G   | G   | G   | G   | G   | N   | N   | T   | C   | T   |
| PAK38    | A   | G   | A   | A   | A   | G   | G   | N   | N   | N   | N   | N   | T   | G   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   |
| PAK39    | A   | G   | A   | A   | A   | G   | T   | C   | A   | T   | T   | G   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | A   | T   | C   | A   |
| PAK47    | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | T   | G   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   |
| PAK48    | N   | N   | N   | N   | N   | N   | N   | T   | A   | C   | A   | T   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   |
| PAK50    | A   | T   | T   | T   | G   | G   | G   | T   | A   | C   | A   | T   | T   | G   | C   | A   | A   | G   | G   | G   | G   | G   | G   | G   | N   | N   | N   | C   | T   | C   |
| PAK52    | A   | N   | N   | N   | N   | N   | G   | A   | C   | G   | T   | T   | G   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | G   | A   | T   | C   | T   |
| PAK54    | C   | G   | T   | A   | A   | G   | G   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | T   | C   | A   | A   |
| PAK58    | A   | N   | N   | N   | N   | N   | N   | T   | A   | A   | A   | A   | T   | T   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | N   | G   | G   | C   | C   | N   | C   |

Grey highlighted cells are private SNPs reported from Pakistan

N represent the missing data

Reference sequence based on the most common genotype observed i.e. Salvador1
5.2.2 Mitochondrial genes analysis

The three genes (cox1, cyt b, cox3) present on mitochondrial genome was analyzed individually to assess the mutations in polypeptide chains.

5.2.2.1 Cox 1 gene

The table 5.4 represents the amino acid mutation in protein chain due to mutation in nucleotides at different regions of cox1 gene. Total 10 amino acid mutations were observed due to variation at 11 nucleotides position. Among 10 amino acids six were replaced by “K”, two were replaced by “N”, one is replaced by “A”. At position 324 of amino acid two consecutive nucleotides mutation was found but there is no effect on amino acid.
Table 5.4 Cox1 Gene: Amino acid mutations observed in the dataset and their respective positions on the nucleotide sequence

<table>
<thead>
<tr>
<th>Position of amino acid mutation on polypeptide sequence</th>
<th>50</th>
<th>55</th>
<th>97</th>
<th>102</th>
<th>125</th>
<th>151</th>
<th>179</th>
<th>219</th>
<th>324</th>
<th>395</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corresponding positions of mutation on the nucleotide sequence</td>
<td>3676</td>
<td>3490</td>
<td>3616</td>
<td>3632</td>
<td>3700</td>
<td>3778</td>
<td>3863</td>
<td>3982</td>
<td>4352</td>
<td>4352</td>
</tr>
<tr>
<td>aReference R E E R E D R D H G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAK19 K K K K K N K N . A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAK22 K K K K K N K N . A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAK23 . . . . . . . . A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAK25 . . . . . K . . A</td>
<td></td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>PAK26 K . . . . . . . A</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAK28 . . . . . . . . A</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAK29 . . . . . . . . A</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAK30 . . . . . . . . A</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAK36 . . . . . . . . A</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAK39 . . . . . . . . A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAK52 . . . . . . . . A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAK58 . . . . . . . . A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*aReference amino acid sequence*
5.2.2.2 Cytb gene

Table 5.5 shows the mutation pattern in Cytb gene located on *Plasmodium* mitochondrial genome. Four SNPs were found on Cytb gene. The mutation at base 5367, 5911 and 5931 cause no changes in polypeptide sequence while SNP found at position 5519 replaced the amino acid “A” to “D” in sample Pak 36.

**Table 5.5 Cytb gene: Amino acid mutations observed in the dataset and their respective positions on the nucleotide sequence**

<table>
<thead>
<tr>
<th>Position of amino acid mutation on polypeptide sequence</th>
<th>195</th>
<th>246</th>
<th>377</th>
<th>383</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corresponding positions of mutation on the nucleotide sequence</td>
<td>5367</td>
<td>5519</td>
<td>5911</td>
<td>5931</td>
</tr>
<tr>
<td>Reference</td>
<td>G</td>
<td>A</td>
<td>K</td>
<td>*</td>
</tr>
<tr>
<td>PAK17</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
</tr>
<tr>
<td>PAK19</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
</tr>
<tr>
<td>PAK22</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
</tr>
<tr>
<td>PAK23</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
</tr>
<tr>
<td>PAK25</td>
<td>.</td>
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<td>.</td>
</tr>
<tr>
<td>PAK26</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
</tr>
<tr>
<td>PAK28</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PAK29</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
</tr>
<tr>
<td>PAK30</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PAK36</td>
<td>.</td>
<td>D</td>
<td>.</td>
<td>.</td>
</tr>
<tr>
<td>PAK38</td>
<td>-</td>
<td>.</td>
<td>.</td>
<td>.</td>
</tr>
<tr>
<td>PAK39</td>
<td>.</td>
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<td>PAK47</td>
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<tr>
<td>PAK48</td>
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<tr>
<td>PAK50</td>
<td>.</td>
<td>.</td>
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<td>.</td>
</tr>
<tr>
<td>PAK52</td>
<td>.</td>
<td>.</td>
<td>.</td>
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<td>PAK54</td>
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<td>.</td>
<td>.</td>
<td>.</td>
</tr>
<tr>
<td>PAK58</td>
<td>-</td>
<td>.</td>
<td>.</td>
<td>.</td>
</tr>
</tbody>
</table>

*unknown codon*
5.2.3 Nucleotide substitution pattern

The analysis involved 226 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There was a total of 223 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

Table 5.6 Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>T</th>
<th>C</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>-</td>
<td>8.41</td>
<td>4.95</td>
<td>7.04</td>
</tr>
<tr>
<td>T</td>
<td>8.65</td>
<td>-</td>
<td>9.75</td>
<td>6.93</td>
</tr>
<tr>
<td>C</td>
<td>8.65</td>
<td>16.58</td>
<td>-</td>
<td>6.93</td>
</tr>
<tr>
<td>G</td>
<td>8.78</td>
<td>8.41</td>
<td>4.95</td>
<td>-</td>
</tr>
</tbody>
</table>

Each entry shows the probability of substitution (r) from one base (row) to another base (column) (Tamura et al., 2004). For simplicity, the sum of r values is made equal to 100. Rates of different transitional substitutions are shown in bold and those of transversional substitutions are shown in italics. The nucleotide frequencies are 29.88% (A), 29.06% (T/U), 17.10% (C), and 23.96% (G).

5.2.4 Genetic clustering analysis

Genetic clusters were analyzed by utilizing 224 markers, 18 populations with in global samples of 231 *P. vivax* isolates. Genetic clusters were identified utilizing STRUCTURE and neighbor joining tree NJ. These approaches would provide a comparative overview of applicability and robustness of these methods to interpret the genetic structure of *P. vivax*. All samples or populations were grouped into *priori* geographical clusters according to WHO malaria regions.

5.2.4.1 Neighbor joining phylogenetic tree

An un-rooted NJ tree was constructed for SNP data. One thousand random haploid re-samplings of the dataset were made and the mean of the values (Bootstraps>50%) for main branches are shown on the NJ tree. The tree is later edited by figtree v1.4.3. Significant bootstrap values (>50) were added in the tree. All populations were grouped into clusters based on their geographical
location. Each cluster is represented by specific color in the tree. Nine clades were identified on NJ tree which are labeled as A to I.

The clade A consist of two samples of PNG (Papua New Guinea) with significant bootstraps value. The clade B consisted of 10 samples of eastern Mediterranean region (Pakistani samples) with significant bootstrap value (83). Clade C was observed with strains from Eastern Mediterranean, Southeast Asia and Americas region while no significant bootstrap value was found. On the other hand, clade D consist of 2 samples of belonging to Americas region (Peru) (bootstrap=65).

Clade E comprised samples of two regions including Americas and Southeast Asia. One of sub-clade consist of two Southeast Asian strains was found with significant bootstrap value (65). Clade “F” consist of only two regions i.e., Western Pacific and Southeast Asia. The sub branches were found with significant bootstrap values (>50) including samples of Western Pacific regions.

Another clade “G” consist of samples of three regions including Western Pacific, Southeast Asia and Eastern Mediterranean (Pakistan). In this clade, a sub branch was well-supported by significant bootstrap value (50). The sub-clade of china and Vietnam was found with well-supported bootstrap (75) and along with another sample of Cambodia the three were found with significant bootstrap (76). Another well supported (bootstrap value=52) small sub branch consist of Western Pacific and Southeast Asia samples of *Plasmodium vivax*. The two isolates of Western Pacific were also observed together with significant bootstrap value (62).

Another main branch of the tree was sub divided into two sub-branches which were categorized into Clade “H” and “I”. The clade H consist of two regions Western pacific and Southeast Asia. The largest clade “I” had sub-clades of significant bootstrap values for Western Pacific (82 and 57), Southeast Asia (59) and eastern Mediterranean (Pakistan) (bootstraps=64).
5.2.4.2 STRUCTURE

The program STRUCTURE uses a Bayesian model-based clustering method to infer population structure using MCMC (Markov chain Monte Carlo) scheme. It assumes linkage equilibrium (HWE) and non-linkage between marker loci within populations. The method also identifies migration or admixture events. (Pritchard et al., 2000). For details see section 2.6.1.1 of chapter 2.
The Bayesian approach implemented in STRUCTURE was used to infer population sub-structure \( P. \text{vivax} \) in the dataset with 21 Pakistani isolates and 210 samples of other countries. Value for optimal number of clusters (K) was allowed to vary between 1 to 20 for 5 replicate consecutive runs (for settings see section 2.6.1.1). The mean of the \(-\ln \text{likelihoods} (\ln \Pr(K/X))\) for 5 independent runs was observed to plateau at K=6 (Figure 5.2).

The STRUCTURE plot based on this optimum cluster or sub-population number (Table 5.7) showed a more condensed genetic structure of the dataset. The delta K method identified six sub-populations with 4 admixtures evident between subpopulations. These admixtures were evident between the STRUCTURE population 1, 2, 3 and one at the end of population.

Pakistani \textit{Plasmodium vivax} population was observed between population 1, 5 and Admixture 1, 2 and 4.
Table 5.7 Composition of clusters observed by STRUCTURE analysis for *P. vivax* populations

<table>
<thead>
<tr>
<th>Populations</th>
<th>Number of samples</th>
<th>Country of origin (Number of samples)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Population 1</td>
<td>55</td>
<td>Salvador (1), Brazil (4), Colombia (3), India (1), Indonesia (3), Madagascar (1), Malaysia (1), Mexico (2), Pakistan (7), Panama (1), Peru (10), PNG (13), Sri Lanka (1), Thailand (7)</td>
</tr>
<tr>
<td>Population 2</td>
<td>55</td>
<td>Cambodia (21), Thailand (30), Vietnam (4)</td>
</tr>
<tr>
<td>Population 3</td>
<td>14</td>
<td>Cambodia (6), China (1), Indonesia (1), Malaysia (3), Thailand (1), Vietnam (2)</td>
</tr>
<tr>
<td>Population 4</td>
<td>67</td>
<td>Cambodia (23), Myanmar (1), Thailand (42), Vietnam (1)</td>
</tr>
<tr>
<td>Population 5</td>
<td>1</td>
<td>Pakistan (1)</td>
</tr>
<tr>
<td>Population 6</td>
<td>2</td>
<td>Papua New Guinea (2)</td>
</tr>
<tr>
<td>Admixture</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Admixture 1</td>
<td>11</td>
<td>Indonesia (1), Malaysia (1), Pakistan (7), Thailand (2)</td>
</tr>
<tr>
<td>Admixture 2</td>
<td>9</td>
<td>Malaysia (1), Pakistan (4), Thailand (2), Vietnam (2)</td>
</tr>
<tr>
<td>Admixture 3</td>
<td>6</td>
<td>Cambodia (3), PNG (1), Thailand (1), Vietnam (1)</td>
</tr>
<tr>
<td>Admixture 4</td>
<td>11</td>
<td>Cambodia (4), Pakistan (2), Thailand (3), Vietnam (2)</td>
</tr>
</tbody>
</table>

5.2.5 Population parameters analysis of geographical clusters

Several parameters were studies for all the geographical clusters by utilizing the SNP data produced from 224 sequencing markers.

5.2.5.1 Genetic diversity and inbreeding indices per geographic cluster

Genetic diversity and inbreeding indices were calculated as described in section 2.5.2.5.3 of chapter 2.

Genetic diversity indices varied across the geographic clusters (Table 5.8). The mean number of alleles was highest in the Malaysia (MNA=2.13) followed by Peru, Brazil, Indonesia and Thailand (2), PNG & Vietnam (1.91), Cambodia (1.87) and Pakistan (1.33). However, a statistic like MNA is subject to sample bias (e.g. estimates are higher for populations with larger sample size). Thus, another sample corrected $H_E$ (Nei’s genetic diversity) was used. It suggested that Malaysia ($H_E=0.52$) and Brazil ($H_E=0.5$) were the most genetically diverse groups. Whereas, Cambodia ($H_E=0.11$) and Pakistan ($H_E=0.06$) were the least diverse groups. These results were only for clusters with polymorphic loci all those with no polymorphic loci were excluded from analysis.
### Table 5.8 Genetic diversity and inbreeding indices per geographic cluster

<table>
<thead>
<tr>
<th></th>
<th>Na</th>
<th>MNAb</th>
<th>MNASD*</th>
<th>LTc</th>
<th>HEd</th>
<th>HE_SD*</th>
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</thead>
<tbody>
<tr>
<td>Sal</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>180</td>
<td>-</td>
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<tr>
<td>Br</td>
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<td>2</td>
<td>0</td>
<td>182</td>
<td>0.5</td>
<td>0.00</td>
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<tr>
<td>Cam</td>
<td>57</td>
<td>1.87</td>
<td>0.34</td>
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<td>-</td>
</tr>
<tr>
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<td>-</td>
<td>-</td>
<td>181</td>
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<td>189</td>
<td>0.26</td>
<td>0.14</td>
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</table>

*SD=Standard deviation
* Number of samples per sub-population
b MNA=Mean number of alleles
c Total number of loci tested. The test was not performed on loci that were monomorphic or not typed for that group
d HE=Mean unbiased (sample corrected) expected heterozygosity (Nei, 1973) calculated overall loci in Arlequin v3.5

Linkage disequilibrium (LD) tests were carried out for non-random association of alleles at different loci) for all loci per sub-population. A statistic called Index of association (IA) was used for this purpose. Since statistic IA is sensitive to the number of loci used in the study, an alternative analogous statistic rbar_d was also calculated. Deviation of IA and rbar_d from zero can be taken as an evidence of LD. A p-value is generated by a random permutation procedure to test the significance of any deviation from zero. Both IA and rbar_d provided clear evidence of significant allelic disequilibrium in Cambodia, Thailand, PNG, Indonesia, Malaysia and Pakistan (p<0.05). Vietnam and Brazil showed non-significant values for IA and rbar_d (P>0.05). The LD test couldn’t
be applied to Salvador, China, India, Madagascar, Mexico, Myanmar, Panama and Sri-Lanka due to small sample size (N=2 or <2).

Table 5.9 Tests for linkage equilibrium/disequilibrium per sub-population

<table>
<thead>
<tr>
<th>Geographical clusters</th>
<th>Ia</th>
<th>rbar</th>
<th>P-value</th>
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<td></td>
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<tr>
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<tr>
<td>Peru</td>
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<td></td>
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<td>PNG</td>
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<td>0.001</td>
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<tr>
<td>Brazil</td>
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<td>1</td>
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<td>0.01</td>
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</tr>
<tr>
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</tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>China*</td>
<td>*</td>
<td></td>
<td>*</td>
</tr>
<tr>
<td>India*</td>
<td>*</td>
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</tr>
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<td>Sri Lanka*</td>
<td>*</td>
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<td>Madagascar*</td>
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<tr>
<td>Myanmar*</td>
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</tr>
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</table>

Grey highlighted cells represent sub-populations in significant LD

*Measures of multilocus linkage disequilibrium calculated by comparison to 1000 randomizations of null distribution in Multilocus v1.3.

*Test not done on these clusters

5.2.5.2 Gene flow and genetic differentiation across the sub-populations

Analysis of population divergence using the FST statistic corroborates genetic differentiation at regional scale and further finds significant genetic differentiation between the different regions. The magnitude of significant FST values are varies for different regions. Moderate genetic differentiation was found among samples of Western Pacific (Cam & Mly, FST=0.13) compared to those from Southeast Asia (Cam & Tha, FST= 0.07, Tha & Viet, FST=0.13).

Great genetic differentiation (FST =0.15-0.25) was observed between samples of America and western pacific (Br & Cam, Br & Vie, Per & Cam, Fst=0.17-0.23), America and Southeast Asia
(Br & Ino, $F_{ST}$=0.15 Br & Tha, $F_{ST}$= 0.21). Highest level of differentiation was observed between Mexico and Peru $F_{ST}$ =0.86, though both belongs to same region (America), whereas the lowest differentiation ($F_{ST}$= -0.78) was between Panama and Peru belonging to same region, this represent the free interbreeding of the *Plasmodium* population located in the same geographical region.

Pakistani strains showed great to very great differentiation with strains from America (Mexico Brazil, Colombia, Peru), Western pacific (Malaysia, Cambodia and Vietnam), South East Asia (Myanmar, Indonesia, Thailand). Another greater genetic differentiation was observed among samples of same geographical cluster i.e. Southeast Asia (Indonesia and Thailand, $F_{ST}$=0.39), though located in the same geographical region.
Table 5.10 $F_{ST}$ estimates of the inter-cluster differentiation in the lower triangular matrix

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<th>Distance method: pairwise difference</th>
<th>Sal</th>
<th>Br</th>
<th>Cam</th>
<th>Chi</th>
<th>Col</th>
<th>Ind</th>
<th>Ino</th>
<th>Mdg</th>
<th>Mly</th>
<th>Mex</th>
<th>Myn</th>
<th>Pak</th>
<th>Pan</th>
<th>Per</th>
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<th>Vie</th>
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</table>

The upper matrix contains $P$ values generated from 1000 random permutations leading to an $F_{ST}$ value larger than or equal to that observed. Dark grey highlighted cells represent $F_{ST}$ values $> 0.15$. Light grey highlighted cells represent $F_{ST}$ values $0.05 < F_{ST} < 0.15$. Significant $F_{ST}$ values ($p < 0.05$) are in bold italics.

**Significant $P$ values**

- **Sal**: Salvador
- **Br**: Brazil
- **Cam**: Cambodia
- **Chi**: China
- **Ind**: India
- **Myn**: Myanmar
- **PNG**: Papua New Guinea
- **Col**: Colombia
- **Ino**: Indonesia
- **Mdg**: Madagascar
- **Pan**: Panama
- **Per**: Peru
- **Srl**: Sri Lanka
- **Mex**: Mexico
- **Mly**: Malaysia
- **Vie**: Vietnam

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5.2.5.3 Estimation of evolutionary divergence by number of differences method

Among all populations PNG showed the highest evolutionary distance with other populations (9-17). Highest divergence value was found between china and PNG (17) followed by Vietnam & PNG (14.08). Pakistani *Plasmodium* population also showed the high divergence with other populations including Vietnam, PNG, Indonesia, Brazil, Malaysia, Thailand, China and Myanmar (3-9).
Table 5.11 Estimates of Evolutionary Divergence over Sequence Pairs between Groups

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<td>3.42</td>
<td>4.28</td>
<td>5.06</td>
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</table>

The number of base differences per sequence from averaging over all sequence pairs between groups are shown. Standard error estimate(s) are shown above the diagonal. The analysis involved 226 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There was a total of 223 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016). Upper matrix shows the standard error while lower matrix shows the distance estimation value. 100 bootstrap replications were run for this analysis.

Viet: Vietnam  Br  Brazil  Per:  Peru
PNG: Papua New Guinea  Mly  Malaysia  Myn:  Myanmar
Srl: Sri Lanka  Tha  Thailand  Pan:  Panama
Ino: Indonesia  Mdg  Madagascar  Col:  Colombia
Ind: India  Chi  China  Mex:  Mexico
Pak: Pakistan  Cam  Cambodia
5.2.5.4 Analysis of Molecular Variance (AMOVA)

AMOVAs was undertaken in the *Plasmodium* groups to test the proportion of total genetic variance accounted for geographic population sub-division. In this case 32.58% variations occurred at intra-population level while 67.42% variation occurred at inter-population level.

Table 5.12 Analysis of molecular variance (AMOVA) undertaken for *Plasmodium* sub-populations as defined by geography

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>D.F.</th>
<th>Sum of squares</th>
<th>Variance components</th>
<th>Percentage of variation</th>
<th>Average F&lt;sub&gt;ST&lt;/sub&gt; over all loci</th>
<th>P&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grouped by Geography</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Among populations</td>
<td>17</td>
<td>345.792</td>
<td>1.61514 Va&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32.58</td>
<td>0.3258</td>
<td>0.0</td>
</tr>
<tr>
<td>Within populations</td>
<td>213</td>
<td>711.845</td>
<td>3.34199 Vb&lt;sup&gt;b&lt;/sup&gt;</td>
<td>67.42</td>
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<tr>
<td>Total</td>
<td>230</td>
<td>1057.636</td>
<td>4.95714</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

<sup>a</sup>Va = Variance component among populations

<sup>b</sup>Vb = Variance component within populations

<sup>c</sup>P value for F<sub>ST</sub> calculated by comparison to a null distribution of 1023 permutations in ARLEQUIN v3.5

5.3 Discussion

Partial or complete sequences of mitochondrial genomes have proved to be useful markers for population studies in a wide variety of organisms (Avise and Walker, 1998, Jorde et al., 1998, Saccone, 1994). In the malaria parasites of the genus *Plasmodium*, the mitochondrial genome is particularly convenient for such studies, since it is only about 6 kb in length and includes only three protein-coding genes: *cox3*, *cox1*, and *cytb* (Wilson and Williamson, 1997). Although *P. vivax* is the main causative agent of malaria outside of Africa, remarkably little is known about the infection dynamics of this pathogen under natural circumstances. A genotyping system that can be used in large-scale studies are urgently needed to strengthen the research on control of *P. vivax* (Koepfli et al., 2009).
The present study analyzed mitochondrial genome data from 224 SNP markers for 231 isolates (including the clinical filter paper Pakistani samples). These included samples typed at LSHTM (N=231 including 21 Pakistani clinical filter paper samples).

The mitochondrial genome organization is perfectly conserved among 23 Plasmodium species (Hikosaka et al., 2011), and there is high (84%-99%) pairwise sequence similarity between these 23 species. In general, mitochondrial genomes show a tendency toward size reduction or deletional bias (Andersson and Kurland, 1998). The high degree of conservation of these mitochondrial genome structures may be attributed to structural constraints which avoid the deleterious effects of reduction and rearrangement on their genome functions. In addition, the high structural conservation may be due to short evolutionary distances, i.e., not enough time for accumulating rearrangements (Hikosaka et al., 2013).

The SNP were observed with comparing the sequences with References Strain Salvador I. Among 224 SNPs data of 18 populations, 37 were found in cox1 gene while 20 and 17 were found in Cytb and Cox3 gene respectively. Among Pakistani sequences 32 SNPs 28 were found as private SNPs. Among private SNPs 4 deletions and only one insertion was found. P. vivax has a highly polymorphic genome that may present some challenges for drug and vaccine development. In fact, SNPs appear to be present at a higher frequency in the P. vivax genome than in P. falciparum genome (Avise and Walker, 1998).

Several characteristics features; such as high mutation rate (Brown et al., 1979), maternal inheritance (Creasey et al., 1994), and lack of recombination (Elson et al., 2001) have made the mt genome an ideal extra-nuclear genome to reconstruct evolutionary histories of the species. Moreover, the mt genome of parasites evolves neutrally and shows no signs of recombination or selection (Joy et al., 2003), hence the whole genome behaves as a single locus and all sites share a common genealogy, which makes it ideal for studying within species variations and phylogenetic analysis. Amino acid mutation among three mitochondrial genes i.e., cox1, cox3 and cytb was observed in this study. In cox1 gene amino acid Arginine (R) is replaced by Lysine (K)
and Glutamic Acid (E) is replaced by Lysine (K) at three positions. Aspartic Acid (D) is replaced by Asparagine (N) at two places, and Glycine (G) is replaced by Alanine (A) at one place. In Cytb gene only one nucleotide was found to be responsible for substitution of amino acid Alanine (A) by Aspartic acid (D). In cox3 gene 17 SNPs were observed but no changes were found in polypeptide chain. In malaria parasites, mt genome is of relevance, due to (i) its small size (~6 kb), (ii) haploid, and (iii) contains 3 protein-coding genes, cytochrome c oxidase I (coxI), cytochrome c oxidase III (coxIII) and cytochrome b (cytb) (Feagin et al., 2012). Three mitochondrial genes are vital for many processes most importantly cytb which is a probable target for Atovaquine resistance (Vaidya et al., 1993).

Deficiency of genetic markers for P. vivax genome has hindered elaborate studies of its population structure avoiding significant contributions in different phenotypes of the parasite such as drug resistance (Rieckmann et al., 1989, Kain et al., 1993, Hastings and Sibley, 2002, Nayar et al., 1997, Cogswell, 1992). SNPs have received considerable attention recently because of their potential as markers for genetic mapping and for studying molecular evolution and population dynamics (Sachidanandam et al., 2001, Reich et al., 2001).

The genetic variation among Plasmodium species reflects the population history demography and geographic distance, though recombination of nuclear genome disturbs the differentiation pattern while absence of recombination in mitochondrial and apicoplast genome is uniquely informative in tracing of dispersal pattern (Fiser Pecnikar and Buzan, 2014). The geographic differentiation seen in organelle genomes may also be subject to evolutionary forces in addition to genetic drift and migration (Preston et al., 2014).

The geographic origin of Plasmodium isolates has significant role in identifying drug resistance and eliminating malaria. The potential limitation of current mitochondrial bar code is the lack of representation of the Indian sub-continent, Central America, southern Africa and the Caribbean, due to insufficient sequence data from these regions. In addition, there is a need to sample more intensively from East Africa, a region of high genetic diversity, high migration and poor predictive
ability. Once the data gaps are filled, the barcode can be rectified to increase its accuracy in assigning sample origin (Preston et al., 2014). Due to closely related mitochondrial lineages of south Asia, central Asia, Africa, and the Middle East, the tracking of geographic origin of these regions parasites was reported challenging. Extensive genetic admixture of South Asian and east Africa parasite was assumed to be due to high percentage of *Plasmodium vivax* strains which are presently circulating in east Africa has been introduced by South Asian migrants. The availability of dense sampling is recommended for tracing geographic origin of imported infections (Mali et al., 2012, Cullerton et al., 2011, Gupta et al., 2012). To determine the continental origin of the isolates, nearly full length mitochondrial genome sequencing is required as informative polymorphism is scattered over the 6-kb mitochondrial sequence (Ferreira et al., 2007, Orjuela-Sanchez et al., 2010, Van den Eede et al., 2010).

In the present study two different computational approaches were applied to reveal the genetic structuring within *Plasmodium vivax* including STRUCTURE and Neighbor joining tree NJ. In neighbor joining tree some of the clades were found well supported with significant bootstrap values. One of the clade consisting of two samples of PNG were well-supported and strongly differentiated from rest of the populations. This showed the genetic isolation of PNG population from rest of the others. Possibly strains from Papua New Guinea own genetically distinct population due to long-term isolation and lack of gene flow. Some of the Pakistani samples were found in separate group which showed their genetic isolation and limited exchange of genetic material with other population. This differentiation can be result from genetic, spatial or temporal isolation of the isolates. There was also no information about the site of collection of these samples within their countries, thus it is possible that the genetic structuring in time might just be because of large spatial distances between the strains (Rougeron et al., 2017).

The populations belonging to distinct geographical regions were grouped together in same clades which depicts the genetic similarities among different populations while some of the populations of same region were found in different clades displaying the dispersal of population and only
possible way of dispersal is the human migration. A noticeable observation was that clades consisted of American and Southeast Asia strains which are geographically far distant from each other. Global movement of parasites threatens elimination and treatment efficacy. By identifying the global patterns of organelles genome polymorphism, we can explore the level to which *Plasmodium* populations worldwide are inter-connected by international malaria migration (Preston et al., 2014).

Routes of import and origin of Pakistani strains cannot be confidently identified owing to missing geographical links in the data. Although some of its strains were genetically similar those from regions located adjacent or bordering Pakistan. It is very difficult to track the geographic origin of samples from south Asia, central Asia, Africa, and the Middle East as they contained the closely related mitochondrial lineages. Mitochondrial genome from these regions failed to provide population specific DNA barcodes for epidemiological studies. One can assume that a high percentage of *Plasmodium vivax* strains which are presently circulating in east Africa have been probably introduced by South Asian migrants, contributing in an extensive genetic admixture of parasites of these regions. It is recommended that availability of dense sampling is required for geographic racking of imported infections (Mali et al., 2012, Culleton et al., 2011, Gupta et al., 2012). Nearly full length mitochondrial genome sequencing is required to determine the continental origin of these isolates. Despite the relatively low levels of local malaria transmission, populations of *P. vivax* are diverse genetically in the North of Pakistan. High influx of Afghan population in the study area significantly contributed to enhancing the number of genetically modified strain into the area as Afghanistan was visited by multiple countries due to war and conflict situation. Analysis of mitochondrial lineages of *P. vivax* showed moderate levels of genetic diversity and sub-structuring of local parasite populations. These data are consistent with the introduction of *P. vivax* in this Pakistani region from multiple sources and at different times.
This may explain why some parasites cluster together with the lineages from south Asia. However, definitive conclusions may require an additional sampling effort from a vast geographical range.

NJ method is a simple heuristic clustering (strategy designed to explore a sub-set of all possible trees, in the hope that the subset will contain the optimal tree) and thus lacks the ability to optimize a criterion of fit between tree and data (Page and Holmes, 1998a). It uses a so-called “greedy algorithm” that carries out a heuristic search of tree space, where at each step it makes a locally optimal choice to ultimately reach a global optimum tree. However, this greedy approach is weighted towards locally optimal solutions, and these often do not approximate a global optimal solution (which in this case is the best fit NJ tree) due to which NJ method may not produce a best data fitted tree (Guindon and Gascuel, 2003, Eickmeyer et al., 2008).

Another approach was used for genetic clustering of the *P. vivax* population. The STRUCTURE analysis of the 224 SNPs used, detected 6 populations. Two populations were found with strains of diverse geographical origin. The K1 population comprised of more diverged isolates relative to K2 population. STRUCTURE output was in congruence to the NJ tree constructed from the same data. The topology within the tree was not well supported for the major clades suggesting weak genetic partitioning within *Plasmodium vivax* and with its out-groups. The other reason for low bootstrap values in the output from NJ might be the low resolution or diversity of SNPs utilized (low sensitivity).

The genetic diversity index, MNA (mean number of alleles) was highest for Malaysia (table 5.8) followed by Brazil, Indonesia, PNG and Vietnam. This index is subject to sample bias, however, Malaysia, Peru and Brazil having a smaller sample size (N=6, 10 and 4 respectively) had higher MNA values, supporting the genetic richness of these group. These regions have high chances of imported cases as noticeable migration or travel occurs to these countries. Rapid human migration pattern increase the existence of diverse genetic strain in a region (FUNDYGA et al., 2002,
Machado et al., 2010, Thiele et al., 2008). Increased genetic diversity due to imported infections were reported in previous study (Gunawardena et al., 2014).

Gene flow and genetic differentiation between populations was estimated used statistic FST (Table 5.10). Highest level of gene flow was observed between Peru & Panama, belongs to similar region indicates free gene flow among closely located populations. Moderate differentiation was found among population of Western Pacific and between the Western Pacific and Southeast Asia population indicating the significant gene flow among vivax population of both regions. No definitive assumptions can be made about the route of import as the connecting links were not present in this study.

Pakistani Plasmodium vivax population showed strongest genetic differentiation with other region’s P. vivax populations including America (Brazil, Colombia, and Mexico), Western Pacific (Cambodia, Malaysia) and South East Asia (Myanmar and Indonesia) suggesting highly differentiated population of P. vivax in Pakistan. American region is geographically distant from Pakistan but genetic differentiation with less distant regions (western pacific and Southeast Asia) was also observed. This differentiation showed limited gene flow between Pakistan and other mentioned regions, which may be attributed to presence of genetically isolated population in Pakistan. These findings are similar with the results of the study compared the genetic structure of Asian and South American P. vivax population. They reported the greatest genetic differentiation among different continents (Imwong et al., 2007). The lack of similarity between two genotypic phylogenies can also be due to differences in molecular clocks of the markers used e.g. faster mutation rate of mitochondrial DNA as compared to nuclear DNA, in general) (Bastos-Silveira et al., 2012, Tibayrenc, 1999, Gomez-Zurita and Vogler, 2003). Moderate differentiation value was observed between Pakistan and Madagascar suggesting the route of import from Africa to Pakistan. Temporal factor cannot be excluded in this case, as the provided data sets other than Pakistan were may earlier than Pakistani P. vivax population (collected in 2014).
The cluster pairs with least gene flow included; America & western Pacific, Americas & Southeast Asia and Western Pacific & Southeast Asia. Those population with less number of samples showed complete isolation, can probably be explained as sampling bias. Given the small size of some of the clusters, it is unclear whether the lack of genetic difference reflects minimal gene flow or merely low power of the test to detect any exchange for small size clusters.

Estimates of evolutionary divergence over sequence pair between groups was also calculated in this study. The populations of western pacific showed highest divergence estimate with other populations. Eastern Mediterranean region (consist of only on type of population i.e. Pakistani samples) showed high divergence estimate with other regions. China and PNG grouped in same cluster showed highest divergence value as compared other population’s estimates. This corroborated the findings from gene flow analysis by $F_{ST}$.

5.4 Conclusion

Marked genetic differences were found among Pakistani *P. vivax* population. The genetic differentiation show the genetic isolation of Pakistani population and evolutionary divergence also confirm these findings. This study concludes the significant mutation in mitochondrial genome sequences of Pakistani *Plasmodium vivax* population. This study also corroborates the use of filter papers as a source of clinical material for population genetic analysis studies. However, major drawback associated with this method were the allele drop outs due to insufficient DNA in these samples. Future studies would require optimizing these methods and having more appropriate sample collection and storage medium techniques which could enhance the quality of DNA for population genetic studies. Future research (nuclear as well mitochondrial genome studies) should ideally identify the extent of heterogeneity and recombination of *P. vivax* within this region. These observations also emphasize the need to scrutinize the genetic structure of *P. vivax* within Pakistan in general, using new sampling sites. The import route can also be find by exploring the genetic strains found in this region. Further Population genetics analysis of the *Plasmodium vivax* strains
of study area as well as Khyber Pakhtunkhwa and Pakistan would be useful in identifying the imported drug resistant strains of different regions which will be helpful in reducing the over use of anti-malarial drugs as well as reduced the economic burden by introduction of effective drugs against these drug resistant strains.
Chapter 6  GENERAL DISCUSSION

This PhD study was aimed to generate latest information on malaria infection by finding parasite prevalence using different diagnostic methods. The study area is among endemic districts of KP with high API index. The new cases and relapse cases leads to increase prevalence in the area. Most of the individuals suffering from malaria remained suffered for several years with no confirmation that either they are relapse or re-infected cases. The proper follow-up study of that cases is necessary as it contributes to disease elimination process. On each visit, they are prescribed with same antimalarial drugs with no effect their illness. The resistance development, prescription of wrong antimalarial drugs, improper dosage prescription, use of inefficient drugs and improper intake of medicine dosage due to illiteracy are the main possible causes.

To control the diseases, it is necessary to map out the correct situation of the disease burden foci and to search out the dominant *Plasmodium* species in the area. In finding the species prevalence, one of the limitation is the timing of sample collection as some of the months are correlated with the presence of particular species: *P. vivax* peaks between April and September while *P. falciparum* peaks between August and December (Williams and Meek, 2011, DMC, 2007, Mukhtar, 2009), (Khatoon et al., 2010). Highest *P. vivax* infections and lowest *P. falciparum* infection were observed in March for Baluchistan (Yasinzai and Kakarsulemankhel, 2008). The village “Hani kala” in the study area is well known for Falciparum cases (Personal communication) but unfortunately no study has been done yet in that area. Such areas need serious attention of the health authorities to eradicate the fatal *Plasmodium* species. These areas contribute in transmission of fatal malaria infection to the adjacent areas.

Specific targeting foci of infection in the malaria endemic areas most likely to hinder transmitting but also save money and time by only uses control in these areas (Fegan et al., 2007; Bhattarai et al., 2007). Complete and accurate data of the previous malaria situation, a good record of malaria transmission and its burden are key elements of a successful programme. The decision to change
to the next phase of the malaria control or elimination programme is made on the basis of progress made in epidemiological indicators. Monitoring changes in transmission intensity and identification of residual malaria foci is therefore very essential for successful malaria control programme and to concentrate intervention efforts (Wahid, 2013).

In this study, it was difficult to locate the main foci of *Plasmodium* in the study area as most of the patients visited for health services were belonged to catchment areas of the health facilities where collection was done due to easy accessibility. One of the restriction for finding actual disease burden is the treatment seeking behavior and access to the health facilities. In present study, it was commonly observed that many patients also took medicine for their ill family members by just telling symptoms. Many of the individuals didn’t bring their females to the facilities due to social customs and just rely on symptomatic treatment. Treatment taken prior to diagnosis was also asked and most of them have taken antimalarial drugs, this may also the reason of negative diagnostic results as antimalarial drugs hampers the parasite from peripheral blood. The clinical study is not sufficient for finding the actual malaria burdened area identification. For this purpose, well-planed field survey is required which is not done yet in the District Bannu.

The health infrastructure at periphery consist of Rural health centers (RHCs), Basic health units (BHUs), Civil dispensaries (CDs) which provides diagnostic service such as Microscopy for malaria suspected individuals and they also provided antimalarial drugs. Unfortunately, these facilities do not fulfill the purpose for which they are established. The population of the related areas visits these facilities just for taking medicines. Due to lack of essential resources and skilled personnel’s these facilities are utilized just for symptomatic treatment while in some diagnostic facilities where diagnosis of the disease is not appropriate due to lack of expertise which leads to over use or unnecessary intake of antimalarial drugs causing health impairment and economic burden. Some of the cases of misdiagnosis with very poor health condition and low hemoglobin level was observed in this study with false positive or negative results as well as wrong
Plasmodium species diagnosis. It was also observed during this study that the lab technicians recommend some of the cases positive or negative based on physical appearance of the patients. According to them most individuals are IDPs and they came to hospital for free medicine without any disease. In government health facilities of KP almost the same situation exists. Most of the malaria burden can be reduce by enhancing the standard of diagnostic facilities at primary health facilities.

In Bannu region one of the cause of high malaria burden is the war and conflict situation in Afghanistan and FATA region. IDPs from FATA region excessively increased the malaria burden. One of the possible reason of increased transmission during IDPs stay in study area is the presence of asymptomatic carriers among that population as they are immuned for malaria antigen so in case of infection symptoms do not develop in them as they had come from malaria endemic region where they develop immunity due to prolong infection. Owing to the breakdown of health systems, mass population displacements, and resettlement of vulnerable refugees in camps or locations prone to vector breeding, malaria is often a major health problem during war and the consequences of war. According to Rowland, during the initial acute phase of the emergency, before health services become properly established, mortality rates may rise to alarming levels (Rowland, 2001). Clinical diagnosis of malaria is unreliable, microscopy or rapid diagnostic tests must be established as quickly as possible, to improve case management and surveillance (Luxemburger et al., 1997).

In this study, high prevalence of P. vivax in comparison with P. falciparum was observed. Comparable situation is reported from different parts of Pakistan (Khattak et al., 2013, WHO, 2017, Khatoon et al., 2009, Khatoon et al., 2010) other studies conducted in FATA (Khan et al., 2016) KP (Wahid et al., 2016, Khan et al., 2016, Razzaq et al., 2014, Murtaza et al., 2009, Daud et al., 2014), Sindh (Uutra et al., 2010, Soomro et al., 2009, Jan and Kiani, 2001), Punjab (Majeed et al., 2016), Baluchistan (Ahmad et al., 2013). In earlier studies, high prevalence of Plasmodium
**vivax** was reported in temperate areas and as predominant species while small outbreaks of *Plasmodium falciparum* were also reported from KP province and larger outbreak were reported from Punjab province (Hehir, 1927, Swaroop, 1949).

There is insufficient epidemiological data available from Pakistan to accurately assess the prevalence of various types of malaria (Khadim, 2002). Malaria transmission is seasonal, with peaks in summer (June-September) for *P. vivax* malaria and late-summer and winter (August-November) for *P. falciparum* malaria. The malaria endemicity in Pakistan has a negative impact on its socio-economic growth and productivity, as the main transmission season coincides with the harvesting and sowing of the main crops (wheat, rice, sugar cane). The Government of Pakistan is implementing Malaria Control Program (MCP) in 72 malaria endemic districts of Pakistan with the public-sector resources and in 19 highly endemic districts with the support from the Global Fund. The interventions of the current project are based on the National strategic framework for Malaria control. Association for Community Development (ACD) is one of the sub-recipient responsible for project implementation in three districts of Khyber Pakhtunkhwa and five FATA agencies namely; districts Nowshera, Charsadda and Mardan, along with Mohmand, Bajaur, Orakzai, Kurram and South Waziristan Agencies (ACD, 2015).

Along with high malaria prevalence in Pakistan, Baluchistan also accounts for high proportion *P. falciparum* cases due to its dominance in Quetta (Khattak et al., 2013, Yasinzai and Kakarsulemankhel, 2003) and other regions including Zhob, East Baluchistan and Khuzdar (Yasinzai and Kakarsulemankhel, 2007, Yasinzai and Kakarsulemankhel, 2008, Farooq et al., 2008). Greater regional variation exists within Baluchistan province for falciparum prevalence, 15% in Zhob to nearly 90% in Quetta, representing high geographic variation among districts. Different studies conducted in Sindh also reported the regional variation (Noor and Akbar, 2003, Beg et al., 2008, Hayyat et al., 2009). Other studies conducted in KP region showed fluctuating levels of *P. falciparum*, 16% in Buner (Noor and Akbar, 2003) to 25% in Bannu and Abbottabad (Idris et al., 2007, Khatoon et al., 2009).
Migrations across the border added to malaria cases in those areas especially the influx of Afghan refugees between 1979 and 1982 (WHO, 2011a). The movement of Afghan refugees into Baluchistan province, Iran, was estimated to result in a 24-36% increase in the number of malaria cases (Basseri et al., 2010). Due to poor security conditions over the past few years in the region an upsurge in internal displacement may have been a contributing factor (Kakar, 2009, WFP, 2009). This large-scale dispersion of susceptible populations has to some extent transformed the global epidemiology of malaria (WFP, 2009), leading to epidemics. One such epidemic was reported in 2003 in an refugee camp of Pakistan (incidence > 100 cases/1,000 persons/year) (Leslie et al., 2009b). P. vivax dominance in the region is confirmed by many other studies. As per 1999 World Health Report estimation, there are about 72 - 80 million cases of P. vivax malaria each year with highest burden observed in South and East Asia (52%), Eastern Mediterranean (15%) and South America (13%) (Mendis et al., 2001). These statistics are reviewed by an analysis using a combination of malaria epidemiology, historical maps, geographic information systems and information on population densities, environment, and vector limits (Guerra et al., 2006).

In South and Southeast Asia, where most P. vivax malaria occurs, P. vivax accounts for up to 50% of malaria cases with prevalence rates between 1% and 6% of the population (Luxemburger et al., 1996, Tjitra et al., 2008, Zhou et al., 2005). The proportional burden of P. vivax is even greater in Central and South America, reaching 71–81% of all malaria cases. In eastern and southern Africa, the P. vivax burden is comparatively lower, accounts for 5% (6 to 15 million cases annually) of the malaria cases (Mendis et al., 2001). With arrival of Chloroquine Resistant P. falciparum, the number P. vivax cases were reduced but absolute number of P. vivax remain high (Singh et al., 2004). On the other hand, the effective control measures reduce the ratio of P. falciparum to P. vivax infections (Carrara et al., 2006).

During earliest study, Hay and colleagues found 25-40% global burden of non-falciparum malaria (predominantly P. vivax) with 132-391 million cases annually. With the consideration of population density of areas endemic for P. vivax, the number of peoples at risk of infection reaches
to 2.6 billion which is slightly greater than that for *P. falciparum*. True burden of *P. vivax* is greatly underappreciated as 25 million new cases are reported annually (Guerra et al., 2006).

Some studies reported the highest disease burden in young children and infants’ immunity usually developing by 10 to 15 years of age (Cattani et al., 1987). A study conducted in Thailand reported that incidence rates varied from 800-200 per 100 person/years among children (under 5 years) and older adults. Symptomatic and asymptomatic carriage occur in low transmission settings, (Roshanravan et al., 2003) although overall 82% of patients with *P. vivax* parasitemia were still found to be symptomatic (Luxemburger et al., 1997).

In this study, some of the mixed infection cases were detected through RDT while diagnosed as single infection through microscopy. Later their confirmation through PCR was couldn’t performed. Due to poor quality microscopy practices, mixed infections are rarely diagnosed and reported, as confirmed by a recent study carried out in the bordering regions of Afghanistan, Islamic Republic of Iran and Pakistan (Zakeri et al., 2010a). Comparatively low and highly heterogeneous transmission was reported for both *P. vivax* and *P. falciparum* in five Afghan refugee camps in Pakistan (Wahid et al., 2016).

Mixed infections are common in the regions where *P. vivax* and *P. falciparum* coexist yet reported rarely. Most of the surveys usually reports less than 2% and careful studies recorded up to 30% and this percentage can be raised with use of PCR (Mayxay et al., 2004). The host response and development of cross species immunity may significantly be affected by mixed infection with different *Plasmodium* species (Maitland et al., 1997). The severity of the disease caused by *P. falciparum* infection is reduced by *P. vivax* when coexisted together in the same host thus reducing the risk of sever malaria (Luxemburger et al., 1997), decreasing the risk of treatment failure (Price et al., 1997), lowering gametocyte carriage (Price et al., 1999), and reducing the prevalence of anemia (Price et al., 2001). However, in higher-transmission areas and areas of emerging drug resistance, the additive burden of severe malarial anemia and maternal malaria has not been addressed in detail. The potential for *P. vivax* to attenuate falciparum malaria requires further...
characterization, and has significant implications for *P. vivax*-only vaccination strategies, and the deployment of drugs such as chloroquine, which have lost efficacy against *P. falciparum* but still retain it against *P. vivax*.

Consistent with what has been in East Africa, the Middle East, and other parts of Asia (Bell et al., 2005), microscopy failed to detect mixed *P. vivax* and *falciparum* infections, which PCR successfully detected. These findings highlight the challenge of diagnosing and treating malaria, more so in areas where mixed species occur. These findings highlight the challenge of diagnosing and treating malaria, more so in areas where mixed species occur. Models predict that treatment of *P. vivax* alone caused by failed diagnosis of a co-infecting, more lethal *P. falciparum* can lead to a rapid surge in *P. falciparum* parasitemia (Mason and McKenzie, 1999). Inappropriate diagnosis will thus cause higher morbidity and mortality from malaria, enhance the development of drug resistance because of administration of the wrong drugs, and prevent appropriate management of serious fever–causing illness such as bacterial infections, resulting in poor treatment outcomes (Ngasala et al., 2008). Because PCR is untenable as a routine confirmatory diagnostic method in such settings, diagnostic accuracy in this area of mixed endemicity could be improved by supplementing microscopy with rapid diagnostic tests (de Oliveira et al., 2009, Hopkins et al., 2008).

FATA region in KP is at high risk of malaria because of internal displacement due to political unrest, people of such areas face a lot of problems regarding diagnosis and treatment. Lack of knowledge about malaria, unhygienic condition and seasonal variation increase the prevalence of malaria in these areas. Most of the population of FATA and FR region was migrated towards Bannu which were declared as IDPs (internally displaced population) and all health and other facilities were provided in Bannu and nearby districts on emergency basis. But usually patient burden is more in Bannu as in normal routine individuals from FR and FATA regions comes to Bannu for availing health services.
Malaria is endemic in 101 countries, 45 are WHO’s African Region, particularly sub-Saharan Africa, with 41% malaria cases, 20 in WHO’s Americas Region with 3% cases, 14 in Eastern Mediterranean Region with 6% cases, 4 in Europe with 3% cases, 8 in South Asia Region with 23% cases and 9 in WHO’s Western Pan Pacific region, with 24% cases. Malaria is also returning to Central Asia due to poor socio-economic conditions and civil war (Mukhtar, 2009). Pakistan is supposed to be at a high risk of infection due to widespread irrigation, agriculture and prolonged monsoon season (WHO, 2011b). Pakistan is among moderately endemic countries but some districts of Sindh, Baluchistan, FATA and KP are categorized among high endemic areas with high API index. Most prevalent is *P. vivax* malaria but in some areas or districts where falciparum is reported as more prevalent than *P. vivax* (WHO, 2017).

Per recent reports of WHO, the number of confirmed cases reached up to 3 million per year in Pakistan. Still Pakistan is categorized with Afghanistan, Somalia, Sudan and Yemen in the east Mediterranean region which contributes more than 95% of total regional malarial burden (WHO, 2017). Per WHO world malaria report (2017), there is >40% decrease in estimated incidence rate while 20-40% decrease in mortality rate (WHO, 2017). Epidemiological data from different regions of Pakistan are unsatisfactory to exactly charge the occurrence of various types of malaria (Khadim, 2002).

In this study, greater number of microscopy positive samples were found positive through PCR while *P. falciparum* infection was detected through PCR which was not detected through microscopy. Greater difference was found between PCR and Microscopy results and little variation was found between RDT and microscopy. Previous studies conducted to compare PCR and microscopy performance shown the high sensitivity of PCR over microscopy (Okell et al., 2012a, Okell et al., 2009). Three to four time more detection rate was reported by previous studies (Adegnika et al., 2006, Malhotra et al., 2005). Submicroscopic infections in the general population are significant in serving as a reservoir of parasites to drive transmission intensity (Okell et al., 2009). In this study the low sensitivity of PCR as compared to microscopy may be due to some
false positive cases detected through microscopy. This study agrees with previous studies reported that false-negative results by microscopy for *P. vivax* are probably due to very low parasitaemia which is very difficult to detect by routine microscopic methods (Postigo et al., 1998). A study conducted in Tanzania in 16 health facilities, showed wide variation in results (only 65% concordance with national reference Laboratory). False positive smears were commonly reported reflecting the tendency of laboratory staff in detecting *Plasmodium* through microscopy as they report smear as positive when they are in doubt especially in case of symptomatic case (Ngasala et al., 2008). Same tendency was observed in the laboratory staff from where data was collected in this study. The inappropriate cleaning of re-usable slides was previously reported in the study area while also observed in the present study. Due to improper cleaning or artifacts results in the diagnostic errors (Garcia, 2007).

Giemsa microscopy is still regarded as the gold standard and the most suitable diagnostic instrument for malaria control because it is inexpensive to perform, able to differentiate malaria species and quantify parasites (Jonkman et al., 1995). However, microscopy is labour-intensive, time-consuming, requires well-trained (Reyburn et al., 2004), competent microscopists and rigorous maintenance of functional infrastructures plus effective quality control (QC) and quality assurance (QA) (Wongsrichanalai et al., 2007). Microscopic diagnosis has been shown to be insensitive and nonspecific, especially when parasitaemia are low or mixed infections are present (Amexo et al., 2004). Sensitivities and specificities as low as 71–72% have been reported (Arai et al., 1996, Snounou et al., 1993b).

Though all diagnostics means have their own advantages and disadvantages in contributing the parasite information but all used means were compared to find the most effective diagnostic way in high endemic areas. The specificity and sensitivity of the diagnostic means were also calculated to find their effectiveness in parasite identification. The correct diagnosis is the key point in identifying the true malaria cases, their treatment and control of disease. To prevent the development of resistance against antimalarial drugs and misuse of drugs, implantation of
corrective diagnostics in all public and private facilities is essential need. At present time, there is non-serious attitude of public towards the malaria treatment as they do not have knowledge about the outcomes of malarial illness. In the study area malaria is taken as a common disease due to lack of education and awareness.

Data from this survey showed overall 17.66% (359/2033) parasite prevalence detected through microscopy and almost similar percentage was found through RDT. A noticeable difference was found between results of microscopy and PCR results. In this survey PCR results showed 30.54% (95/311) parasite prevalence while for the same samples microscopy showed 24.4% parasite prevalence. Among microscopy positive samples some were PCR negative. While microscopy negative samples were detected positive by PCR indicating sub microscopic infection reported in other studies. While among microscopy and PCR processed samples, only PCR detect falciparum infection. These PCR detected falciparum cases were detected as *P. vivax* in laboratory and prescribed with antimalarial drugs used for *P. vivax*. Historically microscopy has been the backbone of the malarial parasite identification and remains be the reference standard even with its disadvantages and due to some limitations of this technique, easy and quick as well as cheaper alternative techniques requires to be evaluated (Gilles, 1933).

Over 70% of malaria cases in Africa were reported with preliminary self-diagnosis and management at home with traditional remedies and self-prescribed medicine (Amexo et al., 2004). After failure of self-treatment they attend the health facility (Chandramohan et al., 2002) which might affect the performance of the test methods and leads o development of parasite resistance against drugs. Same situation was observed in this study.

It is previously reported that Plasmodial DNA persist for up to 8 days remains after the intake of antimalarial drugs (Kain et al., 1994) which could result into false positive PCR diagnosis. Due to its high cost, complexity, lack of electricity, labor intensiveness and log running time the PCR is not suitable for rural endemic settings (Hanscheid and Grobusch, 2002).
A study conducted in Afghan refugees also reported the comparatively high detection rate of PCR as compared to RDT (Wahid, 2013). Four times greater parasite carriage was detected through PCR as compared to RDT which suggested the presence of asymptomatic carriers (carriers with low level of parasite without symptoms) or sub-RDT infection (parasite level lower than sensitivity of RDT but picked up by PCR). In endemic areas, due to continuous exposure results into asymptomatic carrier which may lead to partial immunity (Staalsoe and Hviid, 1998, Shekalaghe et al., 2007, Sutherland et al., 2007, Roper et al., 1996). A review on the submicroscopic infections hypothesized that in lower transmission areas infections on average are older and more likely developed to sub-microscopic phase, while in high-transmission areas repeated infections and super-infection may results in the increase of average parasite density in infected individuals (Okell et al., 2012a).

Sub microscopic Plasmodium infections is also reported from low endemic regions including Yemen (Bin Mohanna et al., 2007), Solomon Island (Harris et al., 2010), Colombia (Cucunuba et al., 2008), Amazon region of Brazil (Suarez-Mutis et al., 2007), Peru (Roper et al., 2000) and Principe (Lee et al., 2010). Asymptomatic carrier were also reported from high transmission area including Nigeria (EKE and NWACHUKWU, 2006, Achidi et al., 1995), Uganda (Njama-Meya et al., 2004), Ghana (Crookston et al., 2010, Owusu-Agyei et al., 2001), Kenya (Bousema et al., 2004), Senegal (Males et al., 2008, Le Port et al., 2008), Gabon (Klein Klouwenberg et al., 2005, Nkoghe et al., 2011) and from intermediate transmission area such as India, Burma (Richards et al., 2007), Thailand (Coleman et al., 2002) also reported with asymptomatic malaria individuals. The gametocyte prevalence among parasite carrier may be as high as 90% (Bousema et al., 2006).

Many of the previous studies also have shown that microscopy and RDT can misdiagnose the considerable number of individuals with low parasitemia which is considered as a great challenge for malaria elimination and control programmes as the submicroscopic infections can act as a
carrier of malaria transmission through gametocytes thus can act as significant barrier in implementation of elimination and control strategies (Shekalaghe et al., 2007, Sutherland et al., 2007, Roper et al., 1996). The submicroscopic infection can contribute in a similar way as microscopic infection can do (Schneider et al., 2007). Therefore for successful elimination and control programs it is necessary to conduct surveys on asymptomatic individuals in diverse malaria settings has been suggested.

Poor microscopy practice including training and skills maintenance, technique of slide preparation, workload, quality and condition of microscope and standard of essential laboratory supplies can lead to false positive results, false negative results and errors in species identification (Durrhelm et al., 1997, Maguire et al., 2006). Even in the developed countries, expert microscopists are scarce and impaired microscopy based diagnosis in hospital laboratories is common (Milne et al., 1994, Thomson et al., 2000, Johnston et al., 2006).

Poor specificity of microscopy is commonly report in comparison with expert microscopy. Use of low standard chemicals and poor slide preparation generates artifacts (bacteria, fungi, stain precipitation, and dirt and cell debris) which can be misguided for Plasmodium parasite. The normal blood components can also confuse the parasite identification (Stow et al., 1999, Wongsrichanalai et al., 2007). To avoid this situation, improved training, high quality smear preparation and staining is essential. Another factor responsible for false diagnosis is the parasite density (Maguire et al., 2006, McKenzie et al., 2003), which needs microscopists proficiency and increased time and number of fields as the recommended number of fields to be observed is 100-400 to declare slide negative (Trape, 1985). Another aspect of false diagnosis is the errors in species identification. Expert microscopists should be able to differentiate between species even at low parasite density. Sometime a thorough observation of think smear is required for morphologic, differential-diagnostic details such as erythrocyte size, shape, and crenation, characteristic dots in the erythrocyte stroma, pigment structure and color, as well as schizonts.
The errors in identification of *P. vivax* and *P. ovale* is commonly reported (Singh et al., 2004) while failure in identification of two most common species i.e., *P. vivax* and *P. falciparum* is frequent but underreported (Milne et al., 1994, McKenzie et al., 2003). Underreporting mixed species infection is also common (Johnston et al., 2006).

Cross reactivity with rheumatoid factor in blood can results in RDT false positive result but its chances can be reduced by replacement of IgG with IgM (Laferi et al., 1997, Grobusch et al., 1999, Mishra et al., 1999). Cross reactivity can also occur with heterophile antibodies (Moody and Chiodini, 2002). Rare false negative results may be due to mutation or deletion of the Hrp-2 gene. The presence of anti-HRP-2 antibodies in human can also lead to negative results despite of significant parasitemia (Biswas et al., 2005). Prevention of development of control line due to presence of inhibitor in patient’s blood is also reported. Several factors in manufacturing process as well as environmental conditions (humidity, temperature) may affect the performance of RDT (Bell et al., 2006, Mboera et al., 2006).

This study was also aimed to describe the risk factors for malaria exposure and transmission of *Plasmodium* infection and to map out the most significant factors contributing the disease or parasite prevalence in the Bannu district. The factors found significant in this study were Socio economic status, sleeping habit, literacy level, availability of health care facility in living area, apparent health condition, body aches, treatment recommended by doctor, presence of domestic animals. All these factors are also reported significant previously.

In this study, it was asked from patients that either they are recommended for test by doctors or they come by themselves. Treatment recommended by doctor was found significant for malaria outcome and possible reason is that doctor advice malaria test on the basis of their clinical symptoms related to malaria or which are the apparent symptoms of malaria.

Apparent health condition was found significant for reducing risk of infection. The possible explanation for it is that people with good health status indicates the better living, nutritional and
health indicators which showed their superior economic condition and these might explain their lower malaria risk. In contrast to satisfactory health condition the poor health status make the individual prone to infections. In present study, significant number of the individuals visited the government laboratory was with poor health appearance and most of them were also diagnosed with low HB by main pathology lab of the hospital. Most of individuals among them were belonged to lower socio economic status indicating poor living, nutritional and health indicator which explain their higher malaria risk.

Among symptoms body ache was found significant in this study. As the study was clinical so all patients were with complaint of the aches. The possible explanation is that it is one of the symptom of malaria illness. The body ache should be considered significant when diagnosing patients in the study area.

In this study the availability or nearness of health facility was found significant for reducing risk of malaria infection. The possible explanation can be the early detection and effective treatment for malaria infected individuals provided by nearby health facilities. While in some cases the inappropriate intake of antimalarial drugs did not eradicate parasite from the body but just removed from the blood and the intake of drugs were prior to the present diagnosis so parasite was could not detected in the blood which may signify the presence of health care facility in vicinity. In primary health facilities of the study area Artheget (Artemether+Lumafantrine) and chloroquine is provided for malaria treatment. In previous studies dormancy of parasites is reported with Artemisinin derivatives. The asexual blood stage parasites may become temporarily inert or dormant and so survive therapeutic concentrations of anti-malarial drugs (Teuscher et al., 2010, Mok et al., 2015). Some asexual parasites become temporarily dormant within their infected erythrocytes, and these may regrow after anti-malarial drug concentrations decline. Generally, the dormant parasites are present at densities below the level of microscopy detection (White, 2017).

Previous review study of KAB (knowledge, attitude and beliefs) about malaria, suggested the
importance of increasing health awareness, mobilizing the local or community healthcare professionals, for prevention as well as early detection and effective treatment of malaria among people who are at risk. They also emphasize on strengthening health system’s technical and managerial capabilities at all level of primary healthcare systems (Regmi et al., 2016).

In contrast of this study previous studies reported the efforts of government health authorities against malaria as generally poor or inadequate mainly due to poor infrastructure, less qualified healthcare practitioners, lack of faith in government medicines, expensive and poor quality treatment, non-compliant or incomplete treatment (irrational and inappropriate treatment, lack of efficacy of drugs, inadequate surveillance and resource-poor health systems) (Anand et al., 2014, Das and Ravindran, 2010, Joshi and Banjara, 2008, Sundararajan et al., 2013).

Literacy level is found significant in this study as low literacy contributes to vector friendly environment (Coker H et al., 2001) including unhygienic conditions, ignorance of using repellents. Due to lack of knowledge most of the people do not know how to prevent themselves from getting malaria and in case of illness do not know about proper intake of prescribed drugs. Inappropriate usage of antimalarial drugs leads to development of resistant strains of *Plasmodium* and increase the number of asymptomatic carriers. The development of resistant strains is not only harmful for a single individual but also hazardous for the whole population as drug resistant strains does not respond to the antimalarial drugs which are used in routine which put unpleasant impact on health and economic condition of individuals and their families. In previous studies the wide range of antimalarial drug resistance was reported and the chloroquine resistance was reported from Southeast Asia and South America in the late 1950s and in the late 1970s in Africa (Kain, 1995, Warhurst, 2001). Chloroquine resistance malaria is also reported from Pakistan (Khan et al., 2004).

Sleeping inside the room was found significant in this study and the possible explanation for this is the limited access of mosquitoes to the human and the use to repellent and sprays can reduce the biting rate of mosquitoes. In some cases, due to insufficient space the same room is used for
cooking and sleeping, smoke produce during cooking expel the mosquitoes from room. Those who slept outside the room and having domestic animals in the same house are at more risk as the number of vector bite is increased with animal’s presence.

The upper middle class Socio economic status of the individuals was found significant for reducing risk of malarial infection as this class lives in concrete build houses and can afford the preventive measures and treatment expenses. There are number of studies which support the relation of low socio economic condition with increasing malaria risk (Woyessa et al., 2013, Thang et al., 2008, Cook, 2010, Feachem et al., 2010, Arasu, 1992, Oemijati, 1992, Butraporn et al., 1986, Dev et al., 2004, Gamage-Mendis et al., 1991). The explanation of low socio economic status to increasing malaria risk is the ignorance of disease due to poverty (do not visit hospital or physician) which lead to increase of asymptomatic infection which acts as a carrier for transmission of disease. Other possible explanation is living in a house which are more likely to increase contact with the mosquitoes as compared to the houses (cemented floor, iron roof, brick constructed houses) in high socio economic status to limit contact with mosquitoes. Moreover, individual with low SES may be less likely to use antimalarial control measures (to reduce favored access of the mosquito vector to man) due to cost. Previously malaria was regarded as disease of poverty and all poverty related factors (availability of electricity, main source of drinking water, time taken to collect water, toilet facilities and construction material of walls, roof and floor of house) were associated with malaria (Abegunde and Stanciole, 2006, Mendis et al., 2009, Hay et al., 2004). Previous studies showed non-glazed windows as significant factor (Ayele et al., 2012) while in this study it was not found significant.

Keeping animals at home was found significant for increasing malaria risk in this study. The association between malaria outcome and domestic animals was also reported in a study conducted in Afghan refugee camp at Jalozai, KP (Wahid et al., 2016). This finding is also in line with studies conducted in Afghan refugees (Bouma and Rowland, 1995) and Ethiopia (Deressa et
al., 2007, Graves et al., 2009), they shown that sharing home with livestock increased the risk of malaria prevalence. The simple explanation for this increase in Plasmodium prevalence is due to increased amount of CO2 and cattle odors that attract malaria vectors (Wahid, 2013).

This study was the first ever study of P. vivax mitochondrial genome in Pakistan as selected studies has been conducted on some of the nuclear genes of P. vivax. Previous studies conducted in Pakistan were the genetic study on nuclear genome of P. falciparum (Khatoon et al., 2009).

Many of the isolates were found in the same clades despite of their geographic origin. PNG (Papua New Guinea) was found with highly differentiated genetic material indicated the maximum mutation or gene flow in the population. Studies carried out on the genetic arrangements of P. vivax populations have revealed noticeable polymorphism of P. vivax populations associated with movement of populations from border countries. Similar studies on P. falciparum in Pakistan show many infections are monoclonal with allelic and genetic diversity like those reported from other low endemic south-east Asian countries (Ghanchi et al., 2010).

In this study P. vivax population from Pakistan showed from great to very great differentiation from rest of the populations which showed the limited gene flow. High number of SNPs (single nucleotide polymorphisms) were observed in these isolates which showed the mutation within population.

**Conclusion**

It is recommended on the basis of these results, the use of both microscopy and PCR for the characterization of Plasmodium spp. (McManus and Bowles, 1996). Despite of all limitation microscopy should remain a routine diagnostic tool for malaria due to its high specificity as previously observed (Musalika, 2010b) and cost and time effectiveness. However, for malaria epidemiological studies in low endemic area a more sensitive diagnostic tool PCR is recommended.
Based on current study, we also recommend PCR for identifying the species in population-based studies which will assist in establishing of improved associations among epidemiological dynamics. We also recommend the inclusion of PCR technique into national reference centers institutions accountable for malaria control.

There is the need therefore, to expand malaria diagnostic services as part of a greater framework of health system strengthening within resource-limited settings. Increasingly, countries and implementing partners have identified that limited diagnostic capacity represents a major barrier to implementation and sustainability of prevention, treatment and care programs for malaria (Maputo., 2008).

The present findings confirmed the use of RDT for general use when the number of patients is large. However, for more reliable results microscopy and particularly PCR could be employed.

In summary, the data presented here recommend that efficient and continued surveillance is needed to sufficiently capture the current situation on a population level, to reliably assess submicroscopic levels of parasitaemia and to provide information on transmission variation over time. Malaria indicators surveys to measure the prevalence and burden of the disease should be initially population and health facility-based, then narrow down to foci and ultimately to individual cases as malaria control programme gradually shift to elimination programme, as recommended by WHO (WHO., 2007).

Malaria control effectiveness may be improved by identifying foci of ongoing transmission, which can be targeted for more frequent and intensive care. Some of the high burden areas were identified in this study. The information about mosquitoes breeding sites is not obtained which may be one of the reasons of these areas to be in the hot spot. Therefore, it will be useful for control programs to check for potential mosquitoes breeding sites and might apply vector control measures to target outdoor biting vectors for example larviciding of mosquito breeding sites and adult vector (Fillinger et al., 2008).
The areas reported with high malaria prevalence (especially Baluchistan and tribal areas of KP) needs MIS (malaria indicator survey) or other community based survey (to locate actual burden and transmission) as these areas are reported with high malaria burden, as the reported prevalence is on basis of hospital records or passive case detection which may be unreliable and outdated. The study area reported with moderate (66.08%) literacy rate (rank 62) in Pakistan (Naz et al., 2016), therefore awareness about protection from malaria, health seeking behavior, prompt diagnosis and treatment can lead to significantly improvement in malaria control and finally elimination.

Moreover, health status, body aches, availability of health care center, sleeping habit, literacy level, Socio economic status and keeping animals at home was found to play significant role in spread of malaria. The correction or improvement of all these factors in study area can help in reducing the risk and transmission of malaria.
APPENDICIES

Appendix A Peshawar University ethics committee approval

DEPARTMENT OF PHARMACY
University of Peshawar, Khyber Pakhtunkhwa Pakistan

ETHICAL APPROVAL FORM

Applicant Number 06-J-C-16 Pharm
Date of approval 18.03.2016
Name of Principal Investigator Ms. Latima Jahan
Department_Institute Department of Zoology, University of Peshawar
Head of the Department Prof. Dr. Naheed Ali
Name of Supervisor Prof. Dr. Naheed Ali
Name of Co-supervisor Dr. Nazma Habib Khan
Title of Project MALARIA ENDOSCOPY IN DISTRICT BANNU, KHYBER PAKHTUNKHWA: IDENTIFYING GENOTYPES OF PLASMODIUM FIVAX IN THE REGION

This application is approved by the ethical committee

1. Prof. Dr. Muhammad Soeed (Convener)
2. Prof. Dr. Zulfiqar Iqbal (Member)
3. Prof. Dr. Fazal Subhan (Secretary)
4. Prof. Dr. Muhammad Ismail (Member)
5. Prof. Dr. S. Akram Shah (Member)
6. Prof. Dr. Jawad Ahmad (Member)
7. Prof. Dr. Safdar Hussain Shah (Member)
8. Prof. Dr. Fayyaz ur Rehman (Member)
9. Prof. Dr. Sabih Islam (Member)
10. Dr. Hamayun Khan (Member)

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Appendix B Questionnaire Form Administered in the study

Information will be collected from patients suspected malaria. Questioners will be based on the following requirements:

Date: Name:
Gender: Locality:
Age: Education:
Population displacement: (Residents / IDPS / others)…..
Duration of previous illness: ………………………

Previous illness of malaria: Yes/No Previous Fever history:

General health: Poor/Weak/Satisfactory

Domestic animals: Yes/No Screening against malaria: Yes/No

House type: Mud/ Concrete/ Tent

Water reservoir: Yes/No

Sleeping habit: Inside room/Outside room

Blood group: ………………………

Health care facility: Yes/No

Antimalarial drugs intake: Yes/No

Socio economic status: Lower Class/Lower Middle/Upper Middle/Upper class

Symptoms: ……………………………………………

Test diagnosed by doctor: Yes/No

Family members having malaria:

Microscopy result: +ve/-ve Species

RDT Result: +ve/-ve Species
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