

**Assessment of exposure, infection and risk for malaria in
Afghan refugee camps in Khyber Pakhtunkhwa (KP),
Pakistan**

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Abstract

Northern Pakistan, Khyber Pakhtunkhwa (KP) is low malaria endemic area characterised by seasonal transmission with predominantly vivax malaria. Migration of high number of Afghan Refugees in 1978 into KP led to concerns for an increase in malaria, as the malaria incidence in this group was reportedly high compared to the local Pakistani population. Considerable progress has been made in controlling malaria through operational research in the camps where the Afghan refugees reside. However, this process requires effective, repeatable active surveillance tools for monitoring malaria control as availability of accurate data is the major challenge at present.

The aim of this PhD project was to generate current information on malaria infection rates through parasite prevalence and malaria exposure using antimalarial antibody responses. The project also investigated the risk factors of malaria and heterogeneity in the geographic distribution of malaria in the camps by using GIS data with serological responses and parasite prevalence data. As an ancillary objective the project aimed to determine the prevalence of G6PD deficiency in the study population.

A cross-sectional survey was conducted in five Afghan refugee camps of KP between June and September in 2010. Blood samples were obtained on filter paper from 2526 individuals and tested by rapid diagnostic test, parasite species specific PCR and ELISA for antibody responses to *Plasmodium vivax* and *Plasmodium falciparum*. A questionnaire was administered to collect household and individual based information to determine the potential risk factors of malaria.

Heterogeneity in malaria was observed between the studied camps based on seroprevalence, which ranged from 17%-45% for *P. vivax* and 3% to 11% for *P. falciparum*. Variation in *P. vivax* infection prevalence was also detected between the camps, which ranged from 0.4-9% (RDT) and 5-15% (PCR). Variation in the distribution of malaria was also found within the camp using spatial/GIS data with clear foci of infection identified in 4 of 5 camps. The results showed that as expected parasite based prevalence measures (RDT and PCR) are significantly lower than serological measure of exposure. *P. falciparum* infection prevalence (RDT and PCR) and

seroprevalence was found to be extremely low with *P. vivax* infections predominant. Age seroprevalence changes were more pronounced for *P. vivax* than *P. falciparum* and seroconversion rate was strongly associated with parasite rate.

Increasing age and poorly built houses were associated with increasing risk, while staying in the same camp for the last 6 months and using measures to reducing vector biting such as repellents repellent, coils or insecticide spraying were associated with reduce risk of falciparum malaria. The risk of vivax malaria was observed to increase with increasing age, sharing house with cattle and having fever within 24 hours or two weeks and a reduction in the risk was seen in the individuals who reported use of Insecticide treated Bed Nets (ITN) night prior to survey or used self protection measures from vector. The 563C-T polymorphism of G6PD gene was observed in only 2 unrelated individuals out of 505 individuals tested (0.4%). In conclusion, both parasitological and serological measures were able to detect spatial variation in infection and exposure to malaria at the micro epidemiological level within the camp. This data will help to provide beneficial and up-to-date information to manage control activities in the study area.

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List of Abbreviations

ACT	Active case detection
ACT	Artemisinin combination therapy
AMA-1	Apical membrane protein -1
BHU	Basic Health Unit
bp	Base pair
BSA	Bovine serum albumin
CDC	Centre for disease control, USA
CFT	Complement fixation test
CI	Confidence interval
CSP	Circumsporozoite protein
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dichloro-diphenyl-trichloroethane
EIR	Entomological inoculation rate
ELISA	Enzyme linked immunosorbant assay
GIS	Geographic information system
GMEP	Global malaria eradication programme
GPS	Global positioning system
GSH	Glutathione S-Transferase, <i>Schistosoma japonicum</i>
G6PD	Glucose 6 phosphate dehydrogenase
H ₂ SO ₄	Sulphuric acid
HRP	Horseradish peroxidase
HRP-2	Histidine rich protein
IFAT	Immunofluorescence antibody technology
Ig	Immunoglobulin

IHA	Indirect heamaggtination assay
IPTi	Intermittent preventive treatment for infants
IRS	Indoor residual spray
ITN	Insecticide treated bed nets
KP	Khyber Pakhtunkhwa
LLIN	Long lasting insecticide nets
LSA	Liver stage antigen
LSHTM London School of Hygiene and Tropical medicine	
MAP	Malaria Atlas Project
MHC	Major histocompatibility complex
MSP	Merozoite surface protein
MTI	Malaria transmission intensity
Na ₂ HPO ₄	Sodium Phosphate
NaCl	Sodium chloride
NaH ₂ PO ₄	Sodium dihydrogen phosphate
NaHCO ₃	Sodium hydrogen carbonate
NGO	Non-governmental organization
NWFP	North West Frontier Province
OD	Optical density
OPD	o-phenylene diamine
OR	Odds ratio
PBS	Phosphate buffer saline
PBS-T	Phosphate buffer saline, with 0.5% tween
PCD	Passive case detection
PCR	Polymerase chain reaction
<i>P. f</i>	<i>Plasmodium falciparum</i>
<i>P. v</i>	<i>Plasmodium vivax</i>

PR	Parasite rate
QT-NASBA	Quantitative nucleic acid sequence based amplification
RBC	Red blood cells
RBM	Roll back malaria
RDT	Rapid diagnostic test
Rmp	Revolution per minute
RNA	Ribonucleic acid
RT-LAMP	Reverse transcription loop mediated isothermal amplification
RT-PCR	Reverse transcription polymerase chain reaction
SCR	Seroconversion rate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEA	South East Asia
SES	Socio-economic status
WHO	World Health Organization
µg	Micro-gram
µl	Micro-litre
µm	Micro-meter
µM	Micro-molar
°C	Degree centigrade

Chapter One

General Introduction

Chapter 1. Introduction

1.1. Malaria

Malaria is caused by protozoan parasites of the species Plasmodium, four main species can cause human infection: *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*. However, recently an additional simian species, *P. knowlesi*, has been shown to cause malaria in humans transmitted from primate species, which represents the only known animal reservoir of zoonotic human malaria (Singh et al., 2004). Mosquitoes of the Anopheles species are the natural vectors of malaria and 70 of 422 Anopheles species are known to transmit the parasite (Day et al., 1999). Malarial parasites can also be transmitted trans-placentally (Singh et al., 2003, Fischer, 2003, Valecha et al., 2007) and by inoculation of infected blood, as demonstrated by recorded cases of transfusion-transmitted malaria (George, 1911, Mackintosh et al., 2004).

Malaria is an ancient disease, which has persisted in mankind probably since humans existed. A French physician Charles Louis Alphonse Laveran first discovered malarial parasites in the blood of patients by microscopy in 1880. In 1899, Ronald Ross discovered that human malarial parasites were transmitted by anopheline mosquitoes (Cox, 2010).

1.1.1. Life cycle

The malaria parasite has a complex, multistage life cycle occurring within the vector mosquitoes and the vertebrate hosts. In the lifecycle of malaria parasite (Figure 1.1) humans are infected when bitten by a female infectious Anopheles mosquito, which injects a mean of about 15 haploid sporozoites (Rosenberg et al., 1990). It is the first encounter of the immune system with plasmodium but sporozoites are often injected in small numbers and remain in the blood stream for a short period. Hence sporozoites are considered less immunogenic (effective immunity to this stage is less evident) and do not cause disease symptoms in either immune or non-immune persons (Struik and Riley, 2004). Over 5-16 days (the exact time-frame depends on the malarial parasite species) the sporozoites replicate in the liver and are then released as merozoites capable of invading red blood cells (White, 2004). Red blood cells lack MHC receptors allowing the malarial parasite to evade the immune system in the absence of antigen-

presenting MHC molecules. These asexual forms of the parasite in red blood cells undergo further reproduction in 1-3 days (depends on the species), releasing more merozoites as the red blood cell ruptures (Leggat, 2003). This is the stage of infection which leads to most symptoms of malaria (Dietz et al., 1974, Kitchen, 1949) and becomes detectable in blood by microscopy approximately 7-13 days after sporozoite inoculation (Eyles and Young, 1951). Some merozoites develop into male or female gametocytes, which mature while initially sequestered deep in the vasculature (*P. falciparum*) and therefore are not detected in the blood samples. They first become visible on blood slides in a mature infectious form 11-19 days after the appearance of the asexual blood-stage parasites (Nedelman, 1989). Gametocytes may be taken up in a mosquito's blood meal, transmission requiring at least one male and one female gametocyte. In the mosquito gut, the infected human erythrocyte ruptures, freeing the gametocytes, which develop into mature male and female gametes that fuse to form diploid zygotes. The zygote further develops into motile ookinetes that penetrate into the mosquito stomach wall to form oocysts. These oocytes undergo meiosis, producing the haploid sporozoites which migrate to the salivary glands and the mosquito becomes infectious (Leggat, 2003). In *P. vivax* and *P. ovale* a dormant stage (hypnozoites) can persist in the liver and cause relapse by invading the bloodstream weeks or even years later (CDC, 2010).

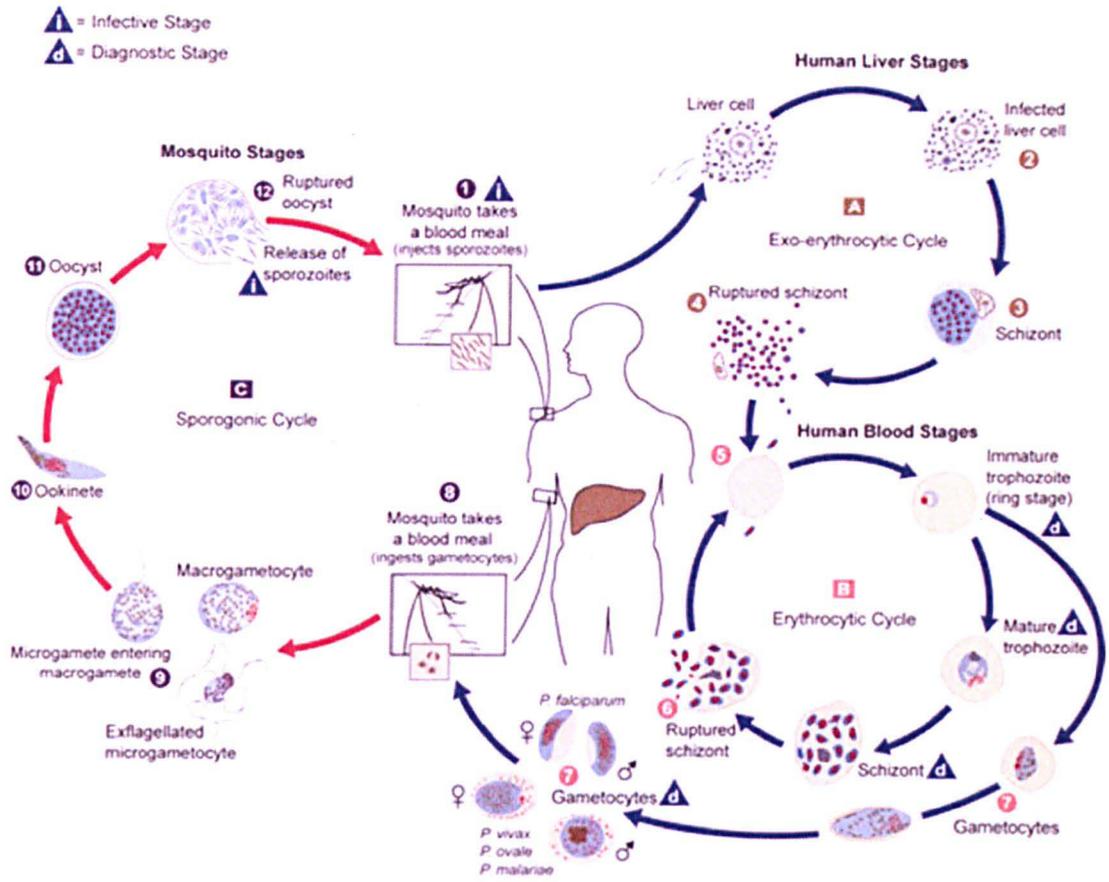


Figure 1.1 The malaria parasite life cycle(CDC, 2010)

Inoculation of sporozoites into the human ① Sporozoites in liver cells ② mature schizonts in liver cells ③ ruptured schizont and released merozoites ④ Dormant stage of *P. vivax* and *P. ovale* (exo-erythrocytic schizogony) **A** erythrocytic schizogony **B** erythrocytic infection by merozoites ⑤ ruptured mature schizonts, releasing merozoites ⑥ Sexual erythrocytic stages (gametocytes) ⑦ The gametocytes ingested by female Anopheles ⑧ sporogony in vector **C** generation of zygote ⑨ Ookinete ⑩ Oocysts formation ⑪ Ruptured oocysts release sporozoites, which move to mosquito's salivary glands to inoculate sporozoites into a new human host ⑫.

1.2. Immune response to malaria

Immunity to malaria is very complex (Langhorne et al., 2008, Augustine et al., 2009), due to several antigenically diverse life cycle stages of malarial parasite (Greenwood et al., 2008). The malaria parasite (*P. falciparum*), has more than 5,300 genes that express some 5,268 predicted proteins, about 2/3 (60%) are suggested to be unique to Plasmodium (different to proteins in other organisms) (Gardner et al., 2002, Gardner et al., 1998, Bowman et al., 1999). These specialized proteins are thought to help in the invasion and growth of parasite within multiple cell types of the invertebrate and vertebrate hosts and to evade host immune responses, and observed to be kept changing often (Florens et al., 2002, Greenwood et al., 2008). Immunity to malaria is widely perceived as transitory such that natural infection with malarial parasites leads to only a partial and short lived immunity such that individuals are not protected against new infections and prone to repeated malaria infections (Struik and Riley, 2004).

In African malaria endemic areas, children (especially non-exposed) are particularly vulnerable and are at high risk of dying from severe malaria (Crawley et al., 2010). In cases of severe pathology it has been observed that protective immunity quickly develops and does not require boosting by re-infection (Struik and Riley, 2004). However, previously exposed individuals to malaria may still be susceptible to uncomplicated malaria (Augustine et al., 2009) until immune effector mechanisms become adept at controlling parasite growth usually after repeated exposure to infections (Struik and Riley, 2004). Asymptomatic infections are thought to persist throughout life even with repeated infections in cases when protective immunity to malaria is incomplete (Wipasa et al., 2010) although the parasite load is lower compared to freshly infected individuals (Struik and Riley, 2004, Okell et al., 2012).

A successful immune response against malaria require a number of inoculations a person receives or exposures to parasite and some intervals between these exposures (Struik and Riley, 2004). In general, acquisition of active immunity to malaria is slow and compounded by age, nutritional/health status and genetics of host. Both innate and adaptive immune responses as well as environmental factors are believed to regulate host immune responses against malaria (Crawley et al., 2010).

1.2.1. Protective immunity against malaria

Division of protective immunity against malaria on the basis of epidemiology:

- Clinical immunity (A): Immunity to protect against severe malaria and to reduce malaria death risk in case of *P. falciparum* (non-cerebral) acute attacks, can be obtained after one or two infections (Gupta et al., 1999)
- Clinical immunity (B): An immune response to reduce the intensity of clinical symptoms which requires frequent inoculations of malarial parasite (Trape and Rogier, 1996, Carter and Mendis, 2002)
- Antiparasitic immunity helps to clear parasite from an infected individual: is effective after many and frequent malaria infections (Struik and Riley, 2004).

The immune system must be able to deal with each parasite species but also parasite strain each time an individual is exposed (Jeffery, 1966, Mendis et al., 1991, Cheesman et al., 2010). In endemic areas, children born to immune mothers are protected against disease during their first half year of life by maternal antibodies and fetal hemoglobin (Amaratunga et al., 2011, Carter and Mendis, 2002). This innate passive immunity is followed by 1 or 2 years of increased susceptibility before acquisition of active immunity (Struik and Riley, 2004). It has been observed that people who lived in endemic areas previously and acquired immunity to malaria by repeated exposure are still vulnerable to uncomplicated malaria but resistant to severe malaria if they inhabit a non-endemic area from months or years (Struik and Riley, 2004). This finding suggests that protective immunity against uncomplicated malaria is short lived as compared to severe malaria.

In naturally acquired protective immunity against blood stage malaria antibodies play an essential part. They protect uninfected RBCs from entry of merozoites, block infected RBC cytoadherence to endothelial cells and increase phagocytosis by monocytes and macrophages (Beeson et al., 2008, Wipasa et al., 2002). However, it is commonly perceived that in the absence of reinfection antimalarial antibodies are short-lived whereby to maintain acquired immunity periodic reinfection is required (Langhorne et al., 2008). One hypothesis is that B cell memory to malaria is defective or suboptimal (Struik and Riley, 2004). A study conducted in Thailand to investigate the induction and maintenance of B cell memory responses to malarial infection has

shown otherwise and demonstrated that B cell responses can be sustained for many years after infection (Wipasa et al., 2010). Another study found 50% antibody positive individuals against malaria in a cohort of Pakistanis and Indians in Bradford who could only have acquired antibodies 10-15 years previously, which indicates that antibody must have been synthesized from B cells, as IgG only lasts approximately one month in the serum following infection (Bruce-Chwatt et al., 1972). Antibodies to gametocytes (sexual blood stages of the parasite, infective to red blood cells and mosquitoes) are not correlated to protection from the disease and they decline with age.

1.2.1.1. Malarial parasite life cycle stages and an body responses

The immune system produces antibodies against each stage of the malarial parasite however the level of antibodies depends largely on the antigen load and the duration for which the immune system remains exposed to the antigen (Cook, 2010). Antibody responses against asexual pre-erythrocyte and asexual blood stage antigens accumulate with age (Smith et al., 1998, Rogier and Trape, 1993, Drakeley et al., 2006), whilst antibodies to gametocyte antigens studied to date do not show this pattern (Drakeley et al., 2006, Graves et al., 1988).

Pre-erythrocyte and sexual stages of the malarial parasite are thought to be less immunogenic and effective immunity to these stages is less evident (Struik and Riley, 2004). Immunity to non-pathogenic sporozoites inoculated by mosquito into human develops after a long exposure to intense malaria transmission. Anti-gametocytic antibodies readily develop with course of natural infection and have some impact on the transmission dynamics of the disease in certain endemic situations (Drakeley et al., 2006, Graves et al., 1988).

Generally, antibodies to asexual blood stage antigens are considered to be the most abundant antimalarial-antibodies due to large numbers of merozoites and continued exposure to antigens (Drakeley et al., 2006). An immuno-epidemiological study has shown a high seroprevalence against many blood stage antigens (e.g AMA-1 or MSP2) as compared to pre-erythrocytic (LSA-1, CSP) and to sexual stage gametocyte (Pfs48/45 and Pfs230) (Bousema et al., 2010b). In seroepidemiological studies the presence of antibodies, which represent exposure to malaria, is more important than the antibodies function in protecting against malaria.

Table 1.1 Level of protective immunity in different age group and endemicity

Types of Malaria transmission	Protective immunity And Age	Geographical location(s)
Stable	<ul style="list-style-type: none"> • High in older age groups • Low in children under 5 	<ul style="list-style-type: none"> • Sub-Saharan Africa
Unstable	<ul style="list-style-type: none"> • Unreliable in older age groups • Absent in children under 5 	<ul style="list-style-type: none"> • Europe and Mediterranean • Asia and Western Pacific • North, Central & South America • Caribbean
Epidemic	<ul style="list-style-type: none"> • Low or absent in all age groups 	<ul style="list-style-type: none"> • Tropical Africa Highland • Central Asia • Caucasus Asia • Latin America

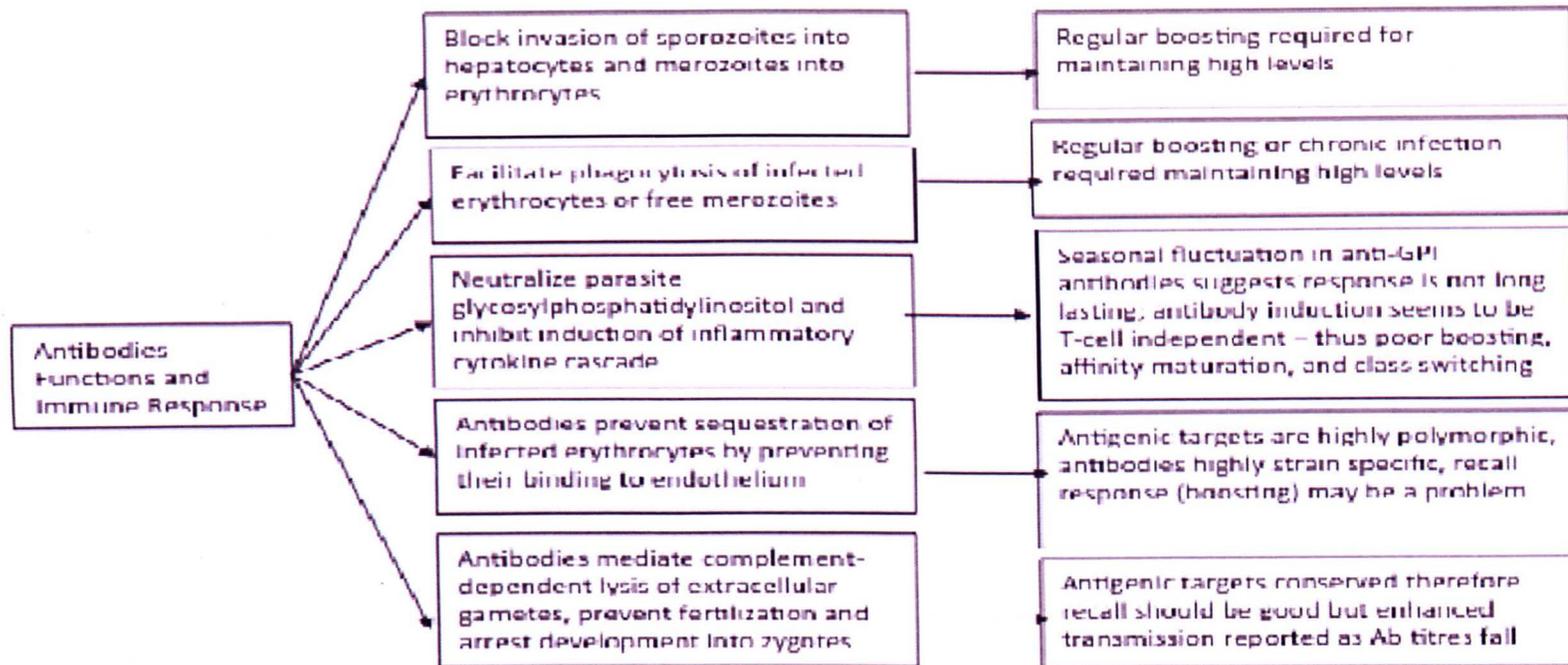


Figure 1.2 Antibody functions and immune response to malaria

Flow-chart showing the likely effector mechanisms of immunity to malaria, adapted from (Struik and Riley, 2004).

1.2.2. Pathogenesis

Clinical malaria is characterized by nonspecific symptoms such as fever, headache, muscle pain, anorexia, nausea, weakness, cough, diarrhoea, abdominal pain, splenomegaly, hepatomegaly, hemolytic anaemia and less commonly respiratory distress. Except for severe anemia, complications such as cerebral malaria, hypoglycemia, metabolic acidosis, renal failure, and respiratory distress followed by coma and death are more common in severe *P. falciparum* infections than infections with other species of malaria (Anstey et al., 2009, Chen et al., 2000, Miller et al., 2002). However, infections with other species of malaria occasionally result in severe clinical symptoms similar to *Plasmodium falciparum* species (Beg et al., 2002), as there are reported many cases of uncomplicated malaria than fatal cases. Although *P. vivax* infections usually cause similar symptoms to the other types of malaria mentioned above, they uniquely cause severe rigors (Crawley et al., 2010).

Falciparum malaria can be severe in residents of all ages of areas with low or no malaria transmission (who have no immunity to malaria). In case of high transmission, severe malaria is more common in young children (Snow et al., 1999, Snow and Marsh, 2002) and pregnant women (Duffy and Fried, 1999, Cot and Deloron, 2003) (whose immunity is decreased by pregnancy, especially during the first and second trimester). Immunosuppressed individuals are at high risk of severe malaria (Grimwade et al., 2004, Grimwade et al., 2003), for example HIV infection in pregnant women was observed to increase the risk of maternal death, miscarriage, stillbirth and neonatal death (Ticconi et al., 2003).

Other *Plasmodium* species especially *P. vivax* (suggested to cause significant morbidity) are generally considered to be relatively benign and rarely life threatening compared to *P. falciparum* (Kitchen, 1949). This view of malariologists remained over several decades but recently life-threatening illness with diagnosis of *vivax* malaria has been observed in endemic settings (Kochar et al., 2005, Anstey et al., 2009). For example, cases of severe *P. vivax* malaria have recently been reported among populations living in (sub) tropical countries including; Brazil (Lanca et al., 2012, Andrade et al., 2010), India (Kochar et al., 2010b, Kochar et al., 2010a), Indonesia (Barcus et al., 2007, Tjitra et al., 2008) and Pakistan (Shaikh et al., 2012).

1.2.2.1. Pathogenesis of Severe Malaria

Several pathophysiological factors including parasite toxic factors such as glycosylphosphatidylinositol (GPI), cytoadherence, rosetting and sequestration, altered deformability and fragility of parasitized erythrocytes, endothelial activation and altered thrombostasis have been found to be involved in the development of severe malaria (Anstey et al., 2009, Chen et al., 2000, Miller et al., 2002). For invasion of *P. falciparum* requires attachment of merozoites to the host cells. In the process of attachment and invasion highly polymorphic proteins (to evade immune system) are involved: those, which help to invade the host cell examples, are AMA1, MSP1, MSP2, and erythrocytic binding antigen (EBA1). Another set of highly polymorphic protein namely *P. falciparum* erythrocyte membrane protein (PfEMP-1) mediates adhesion of infected red blood cells to host cells and sequestration of infected cells.

Invasion and internalization leads to either sequestration (adherence of RBCs infected with malarial parasite to endothelium of capillaries and venules) or rosetting (binding of infected RBCs to uninfected RBCs), which may cause cerebral malaria, hemolysis and malarial complications during pregnancy. In *falciparum* malaria, sequestration of parasitised erythrocytes in the microcirculation and excessive release of proinflammatory cytokines has been implicated as the main cause of disease and death (Planche and Krishna, 2005, Clark et al., 2006). Sequestration of erythrocytes containing mature forms of *P. falciparum* in the microvasculature of vital organs is also a well established feature of fatal malaria in adults and its occurrence in children has been confirmed relatively recently (Taylor et al., 2004).

Sequestration is thought to be mediated by interaction of parasite-derived molecules expressed on the surface of infected erythrocytes (highly polymorphic and clinically variant erythrocyte membrane protein, e.g PfEMP1) (Magowan et al., 2000) and receptors expressed on the surface of the vascular endothelium, of which intercellular adhesion molecule 1 (ICAM1) is probably the most important within the brain (Turner et al., 1994). Tumour necrosis factor (TNF) α was thought to promote cytoadherence of erythrocytes and platelet thrombi within the cerebral microvasculature by up regulation of ICAM1 and other endothelial receptors (Armah et al., 2005, Turner et al., 1994).

1.3. Epidemiology of Malaria

1.3.1. World Malaria Burden

Malaria is thought to be one of the main causes of morbidity and mortality worldwide, about 40% of the world's population lives in malaria transmission endemic areas. About 3.3 billion people were at risk of malaria (WHO, 2011a). Estimated malaria episodes 216 million, of which 81% (174 million cases) approximately occurred in Africa (WHO, 2010b). In 2010, the estimated malaria deaths recorded were 655 000 (91% were in Africa) and more than 80% of these were of children under 5 years of age (Gallup and Sachs, 2010).

1.3.2. Control

Development of dichlorodiphenyltrichloroethane (DDT) in early 1900s, led to an eradication programme from 1955-1972. This was associated with significant reduction in transmission, burden of disease and in eliminating malaria from around 35-40 countries (McKenzie, 2000) and large-scale control of malaria seemed achievable. However, in the late 1960's because of lower funding for malaria and a resurgence of transmission in the late 70s and 80s, the malaria eradication goal of the World Health Organization was switched to control (WHO., 2008b). The resistance of parasite to drugs and mosquito to insecticides is perceived to be one of the problems in malaria control (Toure et al., 2003). Over the past decade malaria funding increased significantly (Snow et al., 2008) and malaria control programs focussed on full-scale elimination and eradication of the parasite instead of management of the disease (Feachem and Sabot, 2008).

1.3.2.1. Malaria control strategies and interventions

There are two major levels of intervention used to control malaria:

1) Vector control

- Long lasting insecticide-treated nets
- Indoor residual spraying
- Larviciding (used in urban areas)

2) Individual treatment with antimalarial compounds (parasite control)

- Intermittent presumptive treatment of malaria in infants, young children and those attending school
- Intermittent presumptive treatment for pregnant women
- Access to diagnosis and effective treatment for children with a fever

First line of defence against malaria is to implement public health control measures to protect people living in malaria risk areas. The recommended control measures depend on the level of malaria risk in an area (Hay et al., 2008, Cohen et al., 2010).

There is evidence that transmission has fallen further in Sub-Saharan Africa in the last few years (Gosling et al., 2008, O'Meara et al., 2008a), although the cause is unclear. Possible reasons for the reported decrease are change in the environment, human behaviour and malaria control interventions. Malaria mortality rates have fallen by more than 25% since 2000, with the largest percentage reductions seen in the European (99%), American (55%), Western Pacific (42%) and African Regions (33%)(WHO., 2008c).

In many of the temperate regions malaria has been successfully controlled and effectively eliminated (Sachs and Malaney, 2002). This has been achieved by standing water reduction, use of insecticides (DDT), changes in agricultural practices, industrialization and improved housing conditions being employed as control strategies (Greenwood and Mutabingwa, 2002).

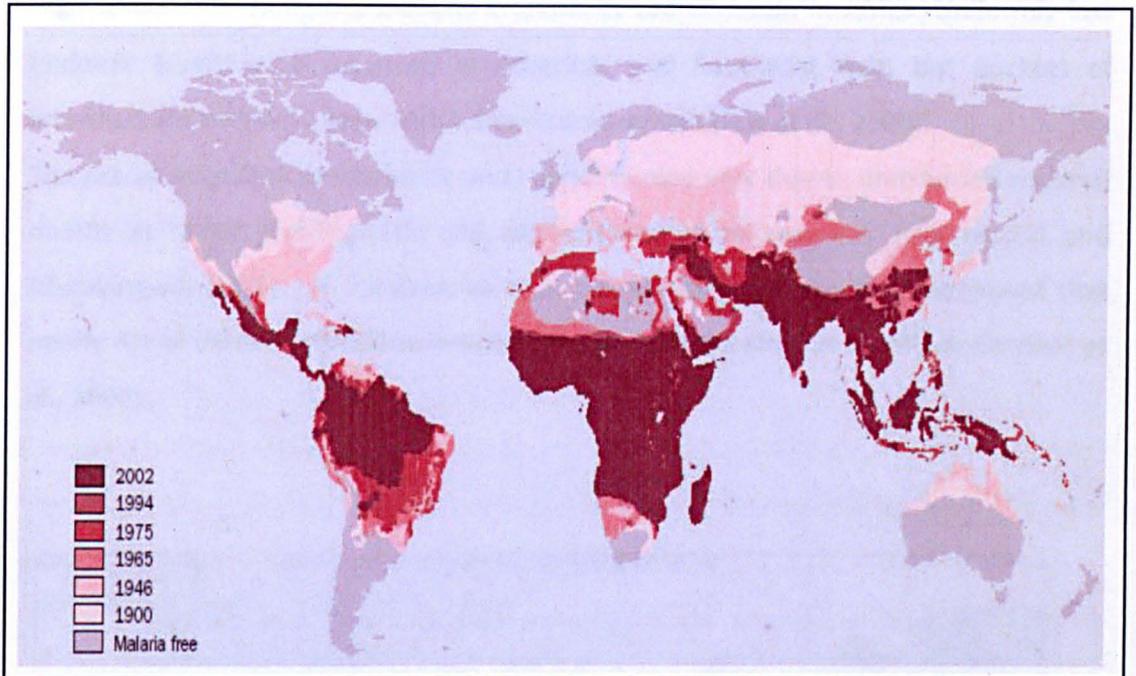


Figure 1.3 Reduction in global distribution of malaria endemic areas over the last century (Hay et al., 2004).

The map shows global distribution of malaria since preintervention distribution (1900) and the years 1946, 1965, 1975, 1992, 1994, and 2002, which illustrates range changes through time. Areas of high and low risk were merged throughout to establish all-cause malaria transmission limits. The 1992 distribution is excluded from the figure for clarity because it was so similar to that of 1994 (Hay et al., 2004).

1.3.2.2. *Plasmodium falciparum*

High levels of *P. falciparum* malaria endemicity are common in Africa. Uniformly low endemic levels were recorded in Americas and Southeast Asia, but pockets of intermediate and very rarely high transmission remain (Hay et al., 2009).

The actual estimates of morbidity and mortality may vary due to unrecorded malarial deaths at home, misdiagnosis and unavailability of microscopes (Greenwood and Mutabingwa, 2002). In medium to high transmission settings it is estimated that nearly 4% of children die of malaria before the age of 5 (Hay et al., 2004, Gardner et al., 2002).

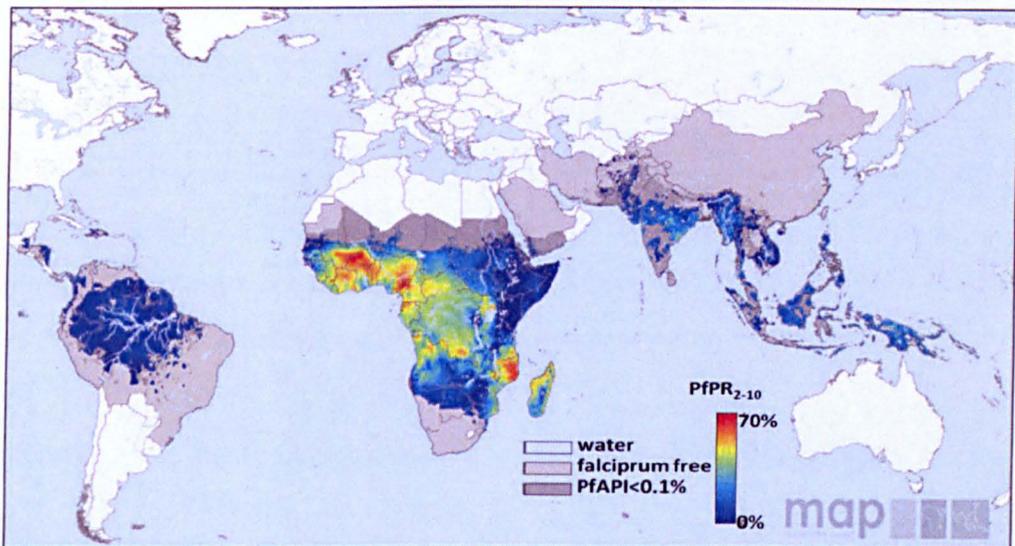


Figure 1.4 The spatial distribution of *Plasmodium falciparum* malaria endemicity map in 2010 globally (MAP, 2013).

This map is showing the age-standardised *P. falciparum* Parasite Rate ($PfPR_{2-10}$) which is estimated proportion of 2-10 year olds in the general population infected with *P. falciparum* at any one time, averaged over the 12 months of 2010 (MAP, 2013).

1.3.2.3. *Plasmodium Vivax*

Plasmodium vivax is endemic in Asia, the Middle East and Western Pacific, with a lower prevalence in Central and South America. It causes more than 50% of all malaria cases outside Africa (Mendis et al., 2001, Sattabongkot et al., 2004). *P. vivax* invariably coexists with *P. falciparum* (often equally prevalent) outside of Africa (Hay et al., 2004). The estimated clinical infections each year by vivax malaria were recorded 72–390 million (Mendis et al., 2001, Price et al., 2007). Vivax malaria is considered to be a benign infection, but it has a significant burden on morbidity and socio-economic status of endemic countries (Mendis et al., 2001).

P. vivax is more challenging to eliminate than *P. falciparum* and the relative proportion of *P. vivax* malaria often increases compared to *P. falciparum* in areas of intensive control measures (Price et al., 2009). This relative increase in vivax malaria is due to relapse caused by the hypnozoites stage as well as the early appearance of gametocytes, the potential of greater transmission even at low parasite densities, and emerging drug-resistant of *P. vivax* in Asia, South America and Africa (Price et al., 2009, Baird, 2009). In many malaria-endemic countries, *P. falciparum* and *P. vivax* has often been observed to be transmitted by the same vector species (Frances et al., 1996, Sattabongkot et al., 2004).

In many malaria-endemic areas (e.g China and Korea) vivax is re-emerging after eradication and becoming a major problem (Chai, 1999, Sleight et al., 1998). For example, in Sa Kaeo (Eastern Thailand province bordering Cambodia) the prevalence of *P. vivax* malaria increased from 20% in 1965 to 50% in 2002, this increase was attributed to changes in the composition and abundance of anopheline mosquito vectors (Sattabongkot et al., 2004). Similarly, in the late 1960s, USA eliminated malaria transmission (Sattabongkot et al., 2004), but re-introduction of *P. vivax* was recorded in Virginia in 2002, where susceptible mosquito vectors were present (2002, Sattabongkot et al., 2004). In these areas the re-introduction of *P. vivax* is mostly linked with the movement of people (travellers, immigrants or soldiers) from malaria-endemic areas (Sattabongkot et al., 2004).

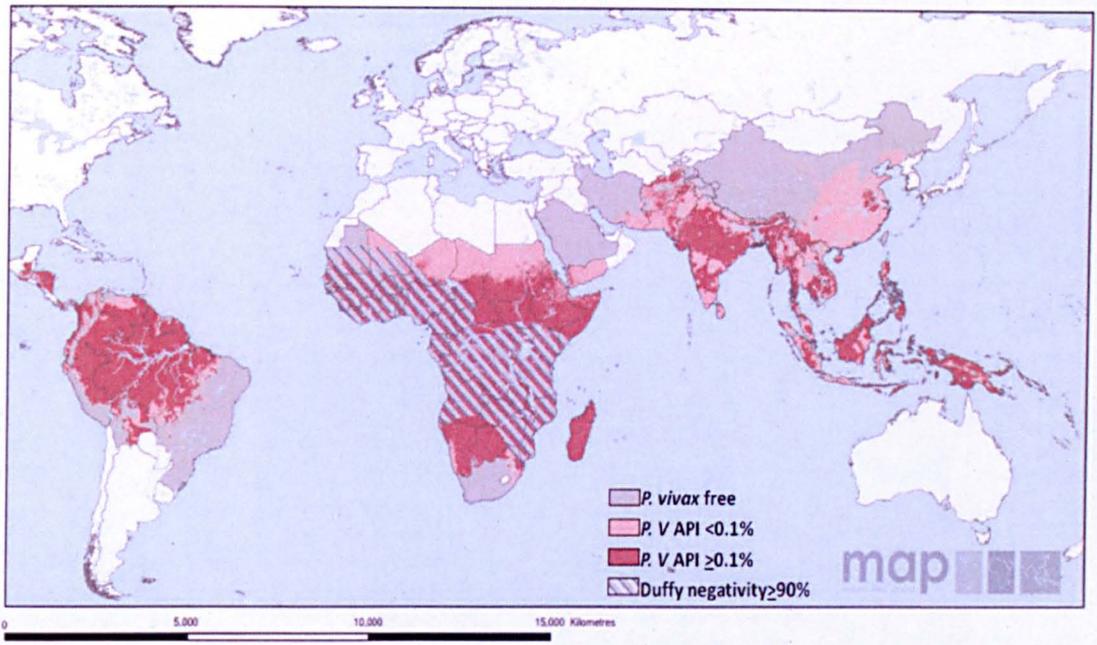


Figure 1.5 Vivax malaria risk defined by PvAPI (MAP, 2009a).

Transmission was defined as:

- Stable (red areas, where PvAPI was recorded greater or equal to 0.1 per 1,000 people)
- Unstable (pink areas, where PvAPI, was recorded less than 0.1 per 1,000)
- No risk (grey areas)

Annual case incidence data over the most recent four years was used.

1.3.2.3.1. Glucose 6Phosphate Dehydrogenase (G6PD) De ciency and its screening signi cance in vivax malaria

In *P. vivax* infections even with effective treatment for acute episodes, one initial infection may lead up to 10 or more subsequent episodes due to its ability to form hypnozoites in the liver (Leslie et al., 2004, Leslie et al., 2008). These hypnozoites acts as a reservoir of infection in host and allow the disease to continue its transmission cycle, which are thought to reduce the efficiency of insecticide treated nets as a control tool. Infected persons may develop further episodes, even if they regularly use an ITN. The radical cure of vivax malaria, using primaquine or other anti-hyozoite drugs, cannot be recommended in the population unless the prevalence of known G6PD (Leslie et al., 2004, Leslie et al., 2008) due to prevalence of G6PD variants with low enzyme activity (Bouma et al., 1995, Ali et al., 2005) in most endemic and formerly endemic countries such as the Mediterranean countries (Turan, 2006). In the presence of primaquine, G6PD deficiency can cause clinically significant and occasionally severe haemolytic anemia (Bouma et al., 1995, Chamchod and Beier, 2013, Leslie et al., 2010). Glucose-6-phosphate dehydrogenase deficiency is an X-linked recessive hereditary defect caused by G6PD gene mutations, resulting in abnormally low levels of glucose-6-phosphate dehydrogenase (G6PD) (Beutler, 1992). The G6PD gene is nearly 18.5kb in length with 1545 bp of coding region (which encodes 515 amino acids) and consists of 12 introns and 13 exons (Szabo et al., 1984, Trask et al., 1991). There are about 140 mutations reported, most of which are point mutations resulting in non-synonymous amino acid substitutions. Secondary mutations may rarely be present in the cistrons (Town et al., 1992, Hirono et al., 2002). The more the non-synonymous mutations, the lower the severity of the disease (Cheng et al., 1999).

G6PD is involved in the pentose phosphate pathway that provides reducing power to all cells in the form of NADPH (reduced form of nicotinamide adenine dinucleotide phosphate). NADPH helps cells to reduce oxidative stress that can be generated by several oxidant agents (Cappellini and Fiorelli, 2008). G6PD is especially important in red blood cell metabolism, as RBCs lack mitochondria and NADPH (of the pentose phosphate pathway) is their only way to protect against oxidative damage (Luzzatto et al., 2001, Williams, 2006).

Neonatal jaundice and acute haemolytic anaemia, caused by an exogenous agent, are the most common clinical signs of G6PD deficiency (Luzzatto et al., 2001). Persistent neonatal jaundice, may lead to kernicterus, which is considered to be the most serious G6PD deficiency problem and can cause acute renal failure (Luzzatto et al., 2001). Most G6PD deficient individuals are asymptomatic. Due to the X-linked pattern of inheritance, mostly males are symptomatic patients, however female carriers can have comparatively less severe clinical symptoms and rarely develop severe acute haemolytic anaemia (Luzzatto et al., 2001, Williams, 2006, Rowe et al., 2002).

Table 1.2 Five clinical classes of G6PD deficiency depending on the severity of the clinical outcome

Class	Clinical Manifestations
I	Severely deficient, chronic non-spherocytic haemolytic anaemia
II	Severely deficient, acute haemolytic anaemia
III	Moderately deficient
IV	Normal activity
V	Increased activity

There are several drugs other than antimalarials, suggested to be harmful to G6PD deficient individuals including, sulfonamides, thiazolesulfone, methylene blue, naphthalene (only when taken at higher doses), analgesics and some non-sulfa antibiotics (nalidixic acid, nitrofurantion, and furazolidone) (Beutler, 1994).

1.3.2.3.2. G6PD deficiency and protection against malaria

Glucose-6-phosphate dehydrogenase (G6PD) deficiency has been hypothesised considered to provide protection against malaria, due to its extensive distribution in malaria-endemic populations. This hypothesis was supported by a haplotypic analysis of the mutant A- and Med alleles of the G6PD gene (Tishkoff et al., 2001). A case-control study conducted in Afghan refugees in Pakistan has shown G6PD deficiency (Mediterranean type) to provide a significant protection against vivax malaria infection whether measured by phenotype or genotype, indicating a possible evolutionary role for vivax malaria in the selective retention of the G6PD deficiency trait in human populations (Leslie et al., 2010). Other previous studies also provided evidence that G6PD deficiency is involved in the clinical protection against malaria in a range of different settings (Mockenhaupt et al., 2003, Ruwende et al., 1995, Mombo et al., 2003). Moreover it has been suggested that the phagocytosis of ring-stage infected G6PD deficient RBCs is twice as high as compared to infected normal RBCs (Cappadoro et al., 1998). The reason for protection against malaria G6PD deficient was suggested the accelerated oxidative membrane damage in deficient cells was defense to the impaired anti-oxidant properties of these cells (Ayi et al., 2004, Williams, 2006).

Many studies to date described the protection against malaria to be related to structural and functional genes of RBC membranes. For instance, negativity of duffy blood antigen encoded by *FY* gene is protective against *P. vivax* malaria. Similarly, the Glycophorin C deficiency (Gerbich negativity) provides protection against EBA-140-mediated invasion by *P. falciparum* parasites (Thompson et al., 2001). CR1 (RBC complement receptor 1) deficient red blood cells infected with *P. falciparum* are shown to have reduced ability form rosettes (Cockburn et al., 2004). Southeast Asian ovalocytosis is also proposed to confer resistance to invasion by a subset of virulent *P. falciparum* parasites and increase adhesion of *P. falciparum*-infected ovalocytes to CD36 (Cortes et al., 2005).

Haemoglobinopathies such as Alpha Thalassaemia have been proposed to provide preferential protection against severe malarial anaemia and also possibly related to reduced surface-expression of CR1 (Wambua et al., 2006, Cockburn et al., 2004). Similarly, HbC homozygotes are shown to give greatest degree of malarial protection,

possibly due reduced surface expression of PfEMP1 in HbC homozygotes (Mockenhaupt et al., 2004, Fairhurst et al., 2005). Hbs homozygotes, on the otherhand, are possibly responsible for accelerated removal of ring-stage infected HbAS red blood cells through improved opsono-phagocytosis and reportedly improve acquisition of malaria-specific immunity in HbAS children (Ayi et al., 2004, Marsh et al., 1989). Similarlry, reduced invasion of HbAE red blood cells (HbE homozygotes) by *P. falciparum* parasites is also demonstrated by in-vitro studies (Chotivanich et al., 2002).

1.3.2.3.3. Epidemiology

An estimated 400 million people worldwide carry a mutated G6PDgene associated with G6PD deficiency. Highest prevalence is reported in Africa, southern Europe, the Middle East, Southeast Asia, the Central and Southern Pacific islands and recently from North and South America and in parts of Northern Europe (Frank, 2005, Howes et al., 2012). Several allelic polymorphs of the gene usually occur simultaneously in areas with high prevalence of G6PD deficiency (Cappellini et al., 1996, Martinez di Montemuros et al., 1997). There are however, some exceptions for instance G6PDA–type is prevalent in about 90% of the tropical regions of Africa. This type is also common in the Americans and in areas with people of African origin such as West Indies.

The second most widely distributed variant of the disorder is the Mediterranean type which is prevalent in the Mediterranean countries (Luzzatto et al., 2001) and some Middle Eastern countries including Israel (most common in Kurdish Jews) (Kurdi-Haidar et al., 1990, Karimi et al., 2003, Oppenheim et al., 1993), India and Indonesia. G6PD A– and G6PDMediterranean are sympatric in several countries along the Persian Gulf (Bayoumi et al., 1996). Seattle and Union variants have been reported in Southern Italy, Sardinia (Fiorelli et al., 1989, De Vita et al., 1989), Greece, the Canary Islands (Cabrera et al., 1996), Algeria, Germany, and Ireland. G6PD Union was also reported in China and Vanuatu Archipelago (Ganczakowski et al., 1995).

Few studies have described the prevalence of G6PD deficiency in Afghanis, whichvaries between 2.9 (Tajiks) to 15.8% (Pathans) (Bouma et al 1995).Some studies have also focused on mandatory testing for G6PD deficiency before administering anti-malarials [e.g. primaquin] in Afghanis. Inspite of high occurrence of G6PD deficiency in the

Afghani as compared to other ethnic groups in Pakistan, very limited data is available about the molecular characterization of G6PD deficiency. Therefore, the present study aimed to identify the G6PD variants in the Afghani population. This will help to provide additional information on G6PD deficiency in Afghanis and help malaria control programmes in the population.

1.4. Malaria endemicity classification

Levels of malaria transmission vary widely both between and within endemic settings (Hay et al., 2000), due to heterogeneity (Variation in the risk of malaria between areas in endemic regions) in transmission malaria endemicity has been variously classified :

Table 1.3 Malaria endemicity based division of transmission areas into classes

Transmission areas categorized classically on basis of endemicity (Pampana, 1969)			Characteristics of the three categories of malaria transmission (Carter and Mendis, 2002)				Recent classification of malaria endemicity (Hay et al., 2008)	
Categories	Transmission characteristics	Parasite prevalence	Type of malaria	Geographical location	Transmission characteristics	EIR	Categories	PfPR2-9
Hypoendemic	Intermittent transmission, unstable, epidemic prone	<10%	Stable malaria	Sub-Saharan Africa	Perennial or seasonal; regular contact between vectors and human hosts	Regular, low to very high	Intense stable endemic	>40%
Mesoendemic	Lower transmission, regular seasonal transmission	10-49%	Unstable malaria	Europe (not endemic presently) and Mediterranean, Asia and Western Pacific, North (not endemic presently), Central, South America and Caribbean	Perennial or seasonal; irregular contact between vectors and human hosts	Irregular, low to medium	Moderate stable endemic	5-40%
Hyperendemic	Intense transmission, no transmission in dry season	50-75%						
Holoendemic	Perennial transmission, little seasonality, high parasitemia level (severe anaemia)	>75%	Epidemic malaria	Highland areas of tropical Africa; Central Asia and Caucasus; Asia and Latin America	Very variable, subject to sudden and rapid change	Rising suddenly, low to medium	Unstable endemic	<5%

1.5. Measuring malaria transmission

Epidemiological patterns and malaria transmission intensity are used to estimate the burden of malaria and to improve strategic interventions for control and elimination (Noor et al., 2011, Cook, 2010). For control programmes it is essential to have regular assessment of malaria transmission intensity to understand the dynamics of malaria transmission in a population, to perceive the magnitude of the problem and help to define when and where the greatest risk occurs and so it leads to the development of appropriate control strategies (Kelly-Hope and McKenzie, 2009).

There is need to establish a regular/periodic epidemiological surveillance system to estimate accurately malaria burden by using epidemiological tools, improve diagnosis and proper treatment for controlling or elimination of malaria. There are a number of methods for quantifying malaria transmission and evaluating the impact of control measures. The following are some commonly used measures of transmission intensity.

1.5.1. Biological measures

1.5.1.1. Entomological inoculation rate

A gold standard measure of malaria transmission is the Entomological Inoculation Rate (EIR), which is the mean number of infectious bites received per person per period of time most commonly a year. EIR is the number of infectious bites per person per unit time, commonly expressed per year. It is the product of the human biting rate and the sporozoite rate: The human biting rate (Ma) is the number of vectors biting an individual over a fixed period of time. 'M' is equal to the number of Anopheles per person and 'a' is the average number of persons bitten by one Anopheles in one day. The sporozoite rate (S) is the fraction of infectious Anopheles (with sporozoites in their salivary glands) present and biting (Shaukat et al., 2010). EIR mainly dependent on proximity of households to larval habitat, domestic animals, human avoidance and defensive behaviour and individual attractiveness, depending mainly on odour or infection status.

Entomological inoculation rate (EIR) is extremely useful and one of the major indices of malaria transmission. EIR values are used to assess the effect of indoor residual spraying (IRS), insecticide treated bed nets (ITNs), and source reduction (SR) on malaria transmission to evaluating malaria control programmes (Shaukat et al., 2010). EIR

works with the calculation of the parasite-infected mosquito pool and its tendency to transmit infectious parasites to the human population. Therefore EIR is considered to be the most direct measurement to assess the impact of vector control programmes.

Although EIR is the gold standard measure of MTI it has some limitations. For example, where sporozoite infection rate is low (low transmission areas) distribution of mosquitoes is very heterogeneous and variations occur in the transmission probability from mosquitoes to humans. Therefore extensive sampling may be needed to surmount the above mentioned limitations which will be very time consuming (Smith et al., 2004). Therefore this index is less useful and lacks precision in low transmission settings simply because it is difficult to find and quantify infected mosquitoes.

EIR data provided by different areas is also not standardized due to use of different protocol for mosquitoes capture, (light, chemical and human landing captures), differences in mosquito infectivity determination methods and seasonal variations, so comparison over different regions is thought to be unreliable. EIR is also not considered as a tool to measure heterogeneity in MTI amongst specific areas or household because it is generated for large areas.

However, EIR is commonly used in some African countries, Kenya, Burkina Faso, Gambia and Tanzania, where MTI is reported high and where well established research labs are available but data is scarce for other African countries (Kelly-Hope and McKenzie, 2009).

As the present study was conducted in an area that has been reported as low malaria endemic it was most interested in the use of tools which are useful for low transmission.

1.5.1.2. Prevalence of parasite

The prevalence of infection is an estimate of the proportion of parasite positive people in an area and is the most frequently used measure of malaria endemicity. It is an indirect indicator of transmission intensity and hence the force of infection in a given setting and most commonly measured by RDT, microscopy and PCR (Beier et al., 1999). Prevalence of infection is suggested to be the direct reflection of inoculations, immunity, and treatment effectiveness in humans as people can be treated based on their microscopy results (individual benefit) (Shaukat et al., 2010).

However, prevalence is not always considered to be a very sensitive outcome measure for measuring transmission especially when parasite densities are low and difficult to detect. Additionally, incidence of infection is difficult to measure directly due to the frequency of super infection and the difficulty of distinguishing these from existing infections even using sensitive molecular methods and cohort studies (Conway, 2007). Parasite prevalence data only represents a point measurement of endemicity and can be affected by season, levels of immunity and antimalarial drugs (Cook, 2010). Another major problem with the parasite rate data is the lack of standardization (skills can vary by laboratory) of techniques by different groups (Hay and Snow, 2006).

1.5.2. Epidemiological measures

1.5.2.1. Incidence data

The incidence of clinical disease episodes can be used as an indicator of morbidity and transmission, which is useful for policy makers and clinicians. However, in areas where asymptomatic parasitaemia is common transmission intensity will be underestimated, on the other hand high transmission may be incorrectly concluded due to over diagnosis of malaria because of its non-specific symptoms (the symptoms can resemble other common diseases e.g. fever) (Chandler et al., 2006, Chandler et al., 2008, Reyburn et al., 2004). The extent to which rates of uncomplicated episodes reflect transmission intensity is also uncertain, particularly comparing between different sites which have experienced different transmission intensities for a period of time and therefore have different immunity levels (O'Meara et al., 2008b). There is some evidence that rates of severe malaria are worst in areas of medium or low transmission, raising concerns about reducing transmission (Snow et al., 1997).

It is also thought that in low transmission areas the population will be less immune, meaning the number of infections that become symptomatic malaria cases will most likely to be high will be more common (Wickramarachchi et al., 2006, Wipasa et al., 2010). On the contrary, several studies conducted in low endemic areas including the Solomon Islands (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) have reported asymptomatic (sub-microscopic) infections of malaria.

Hospital records can be used to estimate transmission but these are highly unreliable, since there is a risk of losing information when large numbers of cases occurring in the

community are not referred to hospital (Tasanor et al., 2006). Transmission can be underestimated and go undiagnosed due to less usage of health facility, depending on use of traditional healers, the distance to and standard of care offered by the health facility (Chandler et al., 2008, Yousif El-Safi Himeidan et al., 2005, Cibulskis et al., 2007, Chilundo et al., 2004, Tasanor et al., 2006). Additionally, false positive malaria cases may arise from clinical suspicion by nurses in resource poor settings lacking microscopes and/or expert microscopists (Font et al., 2001). Furthermore, nurses may not be properly trained for reporting (Kunimitsu, 2009).

Another way, to collect incidence data is active case detection (ACD) or active infection detection, which is usually, used by the researcher during eradication and post-eradication control efforts. WHO defines Active Case Detection as “the survey conducted by surveillance agents to visit every locality in a defined area at regular intervals (usually monthly during the transmission season), to detect fever cases (to test for malaria each suspected person so discovered) by visiting individual house, and treat if positive” (Sanders et al., 2012). When properly carried out, ACD is an unbiased (by health seeking behaviour) estimate of incidence rate and will be suitable in those areas where access to health facilities or treatment seeking behaviour reduces contact with the health system. On contrary, it is an unreliable parameter for measuring malaria incidence when carried out haphazardly. Most Often 100s of slides are taken from a malaria negative person on the basis of which all are declared “negative”. It can therefore underestimate the incidence of malaria. Additionally, in the post eradication era, ACD is an expensive method and can only be done by research teams.

To assess difference in exposure between areas spleen rates are sometimes used (Senn et al., 2010) which is the proportion of children 2-9 years of age with a palpable spleen and is an indirect way of measuring impact of malaria on the spleen. There are some disadvantages related with this measure including variability in the skill of examiners and the occurrence of many causes of splenomegaly. It only represents a point prevalence which can change rapidly with time (Shaukat et al., 2010).

WHO utilizes the primary healthcare facilities baseline data currently to construct the epidemiological malaria profile of a country by case reporting (Kunimitsu, 2009). Since malaria reporting may have errors, the national surveillance systems may vary in reporting malaria and quality (Breman and Holloway, 2007). The accuracy of diagnosis and reporting is crucial in order to improve malaria surveillance and achieve malaria

elimination (Kunimitsu, 2009) since misreporting and miscounting of malaria may result in underestimation or overestimation of the actual malaria burden.

1.5.2.2. Use of serological markers

Sero-prevalence data (ie the prevalence of antibodies to specific pathogens or their antigens) has been used to measure Dengue fever (Egger et al., 2008, Ferguson et al., 1999), Chagas disease (Felicangeli et al., 2003), and measles transmission (Hardelid et al., 2008). In 1970's the same approach was applied to estimate malaria transmission (Mattews et al., 1970). Researchers had used antimalarial antibody responses to measure malaria transmission intensity, to evaluate the effectiveness of control measures, detecting latent foci, to estimate the burden of malaria and to diagnose different species (Corran et al., 2007). Serology was also used to confirm malaria eradication in Mauritius (Franks et al., 2003) and Greece (Bruce-Chwatt et al., 1975). The serological approach also enables measurement and interpretation of slight changes in age-stratified sero-prevalence because of its high sensitivity (Drakeley et al., 2005).

Previous studies have proven that serology is a valuable tool for assessing malarial transmission intensity and correlates with EIR and parasite rates (Cook et al; 2010, Corran et al; 2007). It has some advantages in that as antimalarial antibodies persist longer in contrast to lifespan/density of vector or the half-life of discrete parasite infection. Therefore, sero-prevalence has been suggested to be more accurate as compared to the above mentioned conventional measures of malaria endemicity (Corran et al., 2007) especially in low endemic areas (Bousema et al., 2010c).

Serology has also been used to measure recent transmission changes and is particularly suitable for estimating current force of infection at very low levels of transmission (Druilhe et al., 1986) as the traditional methods based on finding cases or infectious mosquitoes was considered less sensitive at low levels of intensity (Bousema et al., 2010c). Another advantage of serology over above-mentioned measures of MTI, suggested was its ability to identify focal malarial transmission in small geographical areas by integration with household level geographical information system (GIS) in order to allow specific targeting by control measures (Cook et al., 2011).

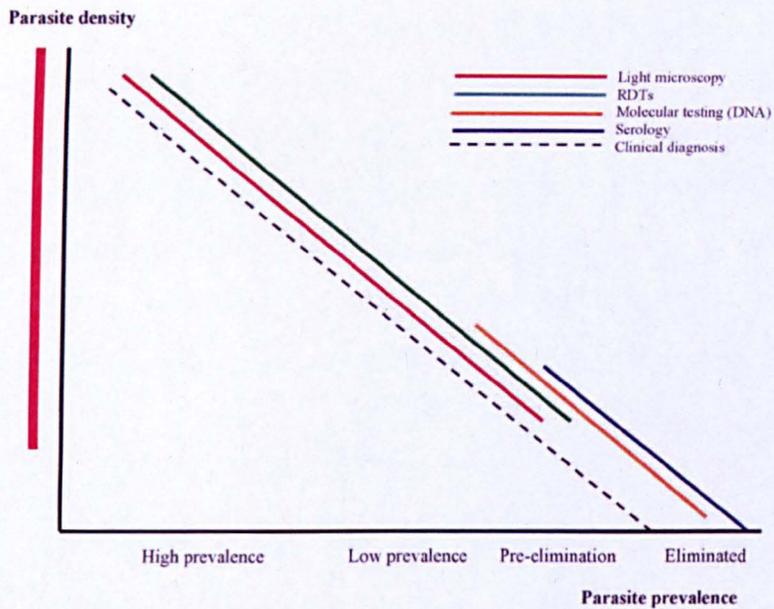


Figure 1.6 Hypothetical graph showing sensitivity of the three tools (EIR, PR and serological markers of exposure) with decreasing or increasing malaria endemicity adapted from (malERA Consultative Group on Diagnoses and Diagnostics, 2011).

1.6. Malaria diagnosis & detection of infection

1.6.1. Microscopy

Microscopy is a widely used method for detection of infection and measuring prevalence of parasite which is relatively quick, cheap and easy to do as compared to molecular techniques (PCR). Microscopic detection of malaria is the current recommended gold standard for its low cost, allowing speciation and detection but it has also some limitations like other diagnostic techniques. One of the problems is that, this method is less sensitive in areas of low transmission because of low overall prevalence and a greater prevalence of low-density infections in the population. Since high parasitemia level is crucial in low transmission areas it will require enough fields to read by microscopy, which will be time consuming and will be difficult to implement in large scale, population-based studies (Rodulfo et al., 2007). Additionally, this procedure requires considerable training of the microscopists to obtain the necessary skills (Corran et al., 2007).

1.6.2. Polymerase chain reaction (PCR)

Low-density infections are thought to be important in maintaining malaria transmission in low endemic areas as well as contributing to the maintenance of acquired immunity (Shekalaghe et al., 2009). It has been observed that the prevalence of malaria parasite infection is higher than that estimated by evaluation of blood using microscope due to presence of these sub-microscopic infections in a population. Therefore it is essential to quantify sub-microscopic infections in a population to understand transmission dynamics for successful decrease in parasite transmission (Okell et al., 2009). Many epidemiological studies consistently provided evidence of prevalence of low-level infection detected through PCR (Okell et al., 2009) because of its higher sensitivity than microscopy for low-density infections (Snounou et al., 1993a, Okell et al., 2009, Musalika, 2010).

PCR was also used to compare the sensitivity and specificity of other non-microscopic methods due to excellent sensitivity and identification to the species level (Mikhail et al., 2011). Currently however, PCR is an impractical routine method in cases of acute malaria diagnosis because of the time involved and the technical expertise required. In addition, the PCR based detection cannot differentiate between new infection and

recrudescence. Another major drawback is that PCR based detection is expensive as compared to other methods for example microscopy, RDT and serology.

Most of PCR-based assays for diagnostic purposes multicopy target genes like 18S subunit rRNA gene or single stranded rRNA (Gunderson et al., 1987, Kawamoto et al., 1996, Singh et al., 1999, Snounou et al., 1993b). Targeting these genes is useful in identifying *Plasmodium* species from clinical samples (on filter papers or from slides) that are commonly producing low parasite DNA content or in cases of low parasitemia (Coleman et al., 2006). The completion of sequencing of the *P. falciparum* genome has highlighted many multigene families e.g. *var*, *rif* and *stevor* genes. The potential of these genes for diagnostic purposes needs to be tested (Gardner et al., 2002).

Conventional nested PCRs are highly sensitive and specific. The most commonly used diagnostic method developed by Snounou and colleagues (Snounou et al., 1993b) uses SU 18s rRNA nested primers that can differentiate between 4 *Plasmodium* species i.e. *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*. However, these nested PCRs require long running periods and there is a high contamination risk. Molecular advancements such as real-time PCR, has been shown to be rapid, highly sensitive and specific, and have low risk of contamination due absence of post-PCR handling. Real-time methods also provide a simultaneous means to quantitate the parasite content. Most of real-time assays target the SU 18s RNA (Andrews et al., 2005, Elsayed et al., 2006, Hermsen et al., 2001, Vo et al., 2007). The greatest disadvantage of real-time PCR is use of expensive consumables. However, Veron et al., (2009) were able to develop a cost effective duplex real-time PCR to detect *Plasmodium falciparum* and *Plasmodium vivax* in the same tube (Veron et al., 2009).

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 has been found useful in malaria detection in the field settings since other commonly used methods like microscopy suffers from low sensitivity in cases of low parasitemia and performance of 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, there are others that report instances of false positives and false negatives by this method. LAMP is prone to errors for visual interpretation of turbidity to test positivity are subject to individual variation in interpretation (Paris et al., 2007).

1.6.3. Rapid diagnostic tests (RDTs)

An increasingly common method to assess prevalence of infection is rapid diagnostic tests (RDT). These are lateral flow immunochromatographic devices that detect protein (antigen most commonly histidine rich protein-II (HRP-2) for *P. falciparum* and lactose dehydrogenase (pLDH) for other plasmodium species) derived from the blood stage of malaria parasites in blood samples (Ochola et al., 2006). Recently a variety of rapid diagnostic tests (RDTs) have been developed which are easy to perform, require less labour and do not need electricity or specific equipment to run (Wongsrichanalai et al., 1999).

For better malaria case management accurate diagnosis was suggested fundamental, whether based on microscopy or RDTs. WHO recommended the antigen-detecting rapid diagnostic tests (RDTs) very essential for malaria diagnosis in areas where good quality microscopy is scarce. Increase in RDTs use has been observed over the past few years for diagnosis and active field studies.

However, previous studies reported that RDTs sensitivity decreases with lower parasitaemia, making these tests unsuitable for individuals with low numbers of parasites (Moody, 2002). On contrary, recent (2008-2011) WHO summary report on RDT performance showed an overall improvement in their performance. Several RDTs detected malaria at low parasite densities (200 parasites/ μ l), had low false positive rates, showed stability at tropical temperatures, were easy to use, and could detect both *P. falciparum* and *P. vivax* (WHO, 2011b). Sensitivity of RDT test is highly dependent on the endemicity of malaria, the parasite density in the target population, differences in transmission levels and also differences in immunity levels that affect the parasite density. For example, in low transmission areas; parasite densities will be lower,

resulting in lower sensitivity of the RDT. To maintain sensitivity of RDT, heat stability in the field (transport and storage of RDTs) was considered vital (Mikhail et al., 2011), especially in areas with high temperatures as compared to areas where temperatures rarely rise above 30°C.

Despite sensitivity, another drawback reported for RDT use was persistence of antigens, in particular histidine rich protein II (HRP-2), in the blood circulation of the patient after parasite clearance, generating false-positive results when microscopy is used as a reference test (Kyabayinze et al., 2008, Tjitra et al., 1999, Moody, 2002). However, other reports stated that the problem is not antigen persistence but the limitations of microscopy, which misses parasite in a sub-set of the patient population (Bell and Peeling, 2006). It has also been known that in some South American areas, parasite did not express HRP2, which suggested that HRP2-detecting tests sensitivity would be reduced in such areas. So in these areas pLDH (*P. falciparum*) has been suggested to be the more effective way to diagnose falciparum malaria (WHO, 2011b). Similarly, genetic variations in HRP proteins can result in variable sensitivities by their RDTs (Kumar et al., 2013). Complete deletion of HRP2 and HRP3 genes in *P. falciparum* has also been reported leading to false negative results by these antigen based RDTs (Kumar et al., 2012, Gamboa et al., 2010, Houze et al., 2011).

1.6.4. Measuring an -malarial an body responses

Sero-epidemiological studies require an ideal malarial antibody detection test that is specific, simple, rapid, easily interpretable and high-throughput (serological surveys to detect and estimate malaria transmission require large sample size). Moreover, the assay should be cost-effective to enable widespread standardized use.

CFT (Complement fixation test) was one of the initial widely used tests to detect malarial antibodies in malaria-infected individuals, as blood slides to detect current infections in case of low parasitemia were found to be less sensitive (Thomson, 1918). The principal of the complement fixation test is based on antigen-antibody complex of interest and an indicator system competition for complement. CFT was simple, convenient, rapid, had minimal demand for reagents and a variety of the test's antigens were readily available. However, because of its major drawbacks including its labour-intensive method, requirement for large antigen quantities and high level of

false positives reported from some studies, the technique was abandoned and replaced by IHA (haem-agglutination test) (Wilson et al., 1975).

IHA was a common method for detecting malarial antibodies since 1960s, based on the principal of cross-linking between antibodies and insoluble antigen to produce blood cells clumping (agglutination). These clumps can be measured by eye (Meuwissen et al., 1974). This method was suggested to be simple and had the added advantage that multiple samples could be tested simultaneously by using micro-titre plates. However, the technique became obsolete due to its non-reproducible results (Meuwissen et al., 1972), standardization difficulties (Bray and el-Nahal, 1966, Lobel et al., 1973), dependency on high concentrations of antibody, reliance on visual examination and low sensitivity (Voller et al., 1974).

Over the years tests used for sero-epidemiological studies have evolved and are meeting some or most of the above mentioned criteria. Some of the commonly used methods are described below.

1.6.4.1. Immuno uorescence an body test (IFAT)

From 1960s onwards the IFAT was one of the widely used serological technique, as it gives results in a few hours and can even be performed with filter paper blood spots and where the antibody is eluted later, which is good for field studies. In this technique, antigen (typically whole parasitized red blood cells) is fixed to glass slide, which is incubated with serum after which a secondary antibody coupled with a fluorescent compound is added which binds to antigen coupled antibodies. A specialized microscope is used to visualize the fluorescence. *Plasmodium eldi* (simian malaria), were initially widely used as an antigen source for IFAT (Voller and Draper, 1982), these were consequently found to be less sensitive in detecting human infections (Draper et al., 1972b, Draper et al., 1972a). Originally infected individuals were used as an isolation source for *P. vivax* and *P. falciparum* antigen, this was later replaced by *P. falciparum* culture.

IFAT was widely used for many years and it is still in use in certain areas (e.g China) (Domarle et al., 2006, Silvie et al., 2002, Zheng et al., 2008). Some previous studies suggested that IFAT displayed higher sensitivity (ability to detect antibodies at a lower concentration) compared to the IHA method (Draper et al., 1972b, Meuwissen, 1974,

Meuwissen et al., 1974). IFAT has also been found to detect higher titres of antibodies than CFT especially at peak infection (Schindler and Voller, 1967).

Drawbacks to using IFAT

There have been reports of cross reactivity of several *P. falciparum* homologues with other plasmodium infecting species particularly rodent infecting species (Brahimi et al., 2001, Ray et al., 1994, Doolan et al., 1996, del Portillo et al., 1991). The method is difficult to standardize (based on visual examination) and vulnerable to user error and bias (differences between users, microscopes and laboratories). Moreover it is a time-consuming process and inter-study comparison are difficult to make due to a wide variety of protocols and reagents in use. Although cultured antigens are available, differences among results were found between different batches of antigens and different storage conditions (Manawadu and Voller, 1978)

1.6.4.2. Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) uses antibodies to detect proteins and immunogens. The ELISA technique was conceptualized and developed by two Swedish scientists Peter Perlmann and Eva Engvall Engvall who published their first paper on the technique in 1971. Voller et al. in 1975 used the first ELISAs to detect malarial antibodies in both *P. falciparum* and *P. vivax* parasite-positive individuals by using antigen obtained from *P. knowlesi* (Voller et al., 1975).

The technique, involves the coating of antigens (most often a single recombinant protein) on to high-binding micro-titre plates. The serum of interest is incubated in the plate, following blocking of non-specific sites. Bound antibodies are then detected with a secondary antibody that is linked with an enzyme. The final step involves the addition of an enzymatic substrate that is then converted if there is bound enzyme present in the well, resulting in colour change or fluorescence measurable by a spectrophotometer.

Advantages

This method is regularly used to investigate humoral responses to malarial antigens in several seroepidemiological studies to look at endemicity of malaria (Drakeley and Cook, 2009). It is a high throughput and easily standardisable (Drakeley and Cook, 2009) method which is relatively cheap and easy to perform. It also generates objective results. Commercially available ELISA components and recombinant proteins help to compare results from different laboratories (Esposito et al., 1990).

1.6.4.3. Protein micro-array

Protein micro-array is a miniaturized assay system to detect antibodies to many antigens simultaneously. This is the most recent addition to the antibody detection techniques and is similar to the ELISA but recombinant proteins are bound to a microscope slide in nanogram quantities. Potentially arrays can screen hundreds of antigens simultaneously and have a greater dynamic range of antibody level detection than ELISA. Limited data are available on their use for sero-epidemiology (Doolan et al., 2008, Gray et al., 2007). Protein microarray is not yet a very common method and it is not routinely used for assessing transmission intensity due to its high cost.

1.6.4.4. Luminex fluorescent assay

High-throughput multiplex assays implemented in Luminex fluorescent microsphere-based technology are preferred for their quick and cost effective screening using minimum sample quantities (Giavedoni, 2005, Nolan and Mandy, 2006, van der Heyde et al., 2007). Each color-coded microsphere set (out of 100 distinct sets) can be coated with analyte-specific capture antibody for the molecule of interest, allowing simultaneous detection and quantitation of reporter signals by Luminex analyzer. The method has been shown to have similar or higher sensitivity compared to ELISA (van der Heyde et al., 2007, Elshal and McCoy, 2006). The assay has been utilized for detection of about 15 vaccine candidates simultaneously in *P. falciparum* (Ambrosino et al., 2010).

1.7. Basic factors that influence prevalence of malaria parasites within a population

1.7.1.1. Vector related factors that influence malaria prevalence

High sporozoite 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 temperature, rainfall and humidity dependent (Adimi et al., 2010).

Temperature: Studies have shown that optimal larval development (optimum temperature range was suggested 25°C-30°C), stable malaria transmission, longevity and feeding frequency of a mosquito are all temperature sensitive (Warrell, 2002). Laboratory based observation had shown that larval mortality increases when water temperature fell below 18°C (Bayoh and Lindsay, 2004). In a study conducted in the Kenyan highlands had shown a small larval survival rate was at low temperatures (Koenraadt et al., 2006), while the adult indoor mosquitoes could survive with temperatures 2 to 3 degrees higher (Garnham, 1945, Protopopoff et al., 2009, Vincke et al., 1946). Moreover speed of development of the parasite within the mosquito was found also temperature-dependent (Warrell, 2002).

Rainfall: In most settings rainfall provides breeding sites for mosquito larvae, though larvae can also be flushed away and killed by heavy rain showers (Paaijmans et al., 2007). This is generally accepted that an increase in humidity may be leading to an increase in adult mosquitoes longevity and consequently the vectorial capacity (Warrell, 2002).

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 metres but recent malaria limit maps have shown malaria endemicity in areas between 1500 and 2000 meters (Baluchistan and KP) (MAP, 2013).

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 afghan refugee camp in Khyber Pakhtun Khawa (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., 2009). 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).

Household and socio-economic: Previous studies have shown that a variety of factors related to household construction such as type of roof, walls, window glazing, open eaves, no separate kitchen, also increase potential to shelter mosquitoes and thus increase malaria transmission (Gamage-Mendis et al., 1991). For example, it is a common observation among the Southeast Asian countries that living in poorly built houses (presence of eaves in the room, thatched roof and non glazed windows) compared to well constructed ones leads to an increase in human-mosquito contact (Butraporn et al., 1986, Oemijati, 1992, Arasu, 1992, Sornmani, 1992).

Other important factors apart from the above mentioned which can effect transmission of malaria in an area include; vector species e.g., zoophilic mosquitos which have particular 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 et al., 1999), as well as whether there are control measures in place (Malikul, 1988).

1.7.1.2. Human related factors that influence malaria prevalence

Population immunity (which is the ability to suppress malaria infections) is thought to be dependent on exposure (Bodker et al., 2006), health status and malnutrition (Shankar, 2000). Similarly, pregnant women were found more susceptible to malaria during pregnancy than non-pregnant women. *Plasmodium falciparum*, which sequesters in the placenta, causes the greatest disease, contributing significantly to maternal and infant mortality (Menendez, 1995). Moreover, the most vulnerable

group is HIV patients (with no or little immunity against the disease) who might enhance malaria transmission and malaria parasite biomass (Protopopoff et al., 2009).

Emergence of antimalarial drug resistance is a huge problem for malaria control strategies, which may play an essential part in the spread of malaria to new areas and in the incidence and severity of outbreaks (Bloland, 2001). Indeed, one of the main reasons suggested for resurgence of malaria in several countries was the emergence and spread of drug-resistant parasites (Bodker, 2000, Trape, 2001). This may lead to progressive build-up of the gametocyte pool in the human reservoir, contributing to increase of transmission (Protopopoff et al., 2009). In South Asian countries, the practice of self-medication (Oemijati, 1992, Arasu, 1992, Fungladda and Sornmani, 1986, Fungladda W, 1991), variety of health-care seeking practices and non-compliance with the prescribed full course of therapy have also been suggested as the cause of occurrence of drug resistance (Fungladda and Sornmani, 1986).

Population migration (Martens and Hall, 2000), reduced access to health systems, poor quality of health services, and socio-economic pressure as population growth (Hay et al., 2004) favourable 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 parasite strains to areas previously free of drug resistance (Bloland, 2001). In Thailand population movement has been suggested as the main reason of the rapid spread of chloroquine-resistant *P. falciparum* strains (Pinichpongse S, 1983).

1.8. Malaria in Asia

The epidemiology of malaria in Asia differs from that of Sub-Saharan Africa where the disease has comparatively higher transmission levels. Although, the transmission is comparatively lower in Asia, the increased burden of the disease has implication for economic activity.

In Southeast Asia, malaria is a major public health issue where 10 out of 11 countries in the region are malaria endemic. The region contains approximately 40% of the global malaria risk population. Maldives is the only country in the region, which is malaria-free of indigenous transmission since 1984. Countries such as Bhutan, Nepal and Sri Lanka have experienced a sharp decrease in the number of reported cases since. The main factor that contributing to malaria transmission in this region is forest associated malaria, accounting for about 40% of total malaria cases and about 60% of the total *P. falciparum* in the region (WHO, 2012b). Change in topography, due to construction and urbanization projects, may give way to malaria susceptible environments. Political unrest and natural disasters resulting in destruction and mass migrations may be another mode of exposing non-immune populations to the disease in endemic areas thus raising concerns of outbreaks. In 2010, in Pakistan post-flood surveys reported approximately 300,000 suspected malaria cases, including confirmed cases of the severe falciparum malaria. The number was above the average annual levels reported (WHO, 2010a). In previous decade (2000-2010), the SEA Region malaria incidence was approx 2.30–3.08 million with around 2400–7000 annual deaths. The highest annual incidence and malaria deaths were reported from India followed by Myanmar and Indonesia, whereas the lowest were reported from Bhutan and Sri Lanka followed by Nepal. The *P. falciparum* prevalence in the regions was estimated to be 45% - 61%. Sri Lanka, Bhutan, North Korea and Thailand showed significant reduction in malaria morbidity. The increase in the proportion of *P. falciparum* in the region may be the result of rising trend of drug resistance, suppressing *P. vivax* incidence by assumptive treatment of all cases with chloroquine, and the use of monovalent RDT (for *P. falciparum* only). On the other hand, the increase might simply be due to the increased case detection in the countries especially in Indonesia, Myanmar, Bangladesh and India (WHO, 2012b).

In Iran, before the implementation of the successful malaria control programme after 1951, about 60% of the total population was living in malaria endemic areas (Edrissian, 2006). Now in Iran, malaria high prevalence has been observed only in the south-east (areas that bordering Afghanistan and Pakistan) contributing 90% of the total cases in the country, with an API 3.6 per 1,000 (Masoumi Asl, 2001, Reza and Taghi, 2011). In Afghanistan, the parasite transmission is seasonal and reported higher in the east of the country bordering Pakistan where the slide positivity reaches 30%. Eighty percent of these cases are *P. vivax* (Leslie et al., 2012).

In China, the annual reported incidence in 2009 was 14,098. Both *P. vivax* and *P. falciparum* are prevalent, although the disease is more concentrated around the southeastern belt neighbouring Vietnam, Laos and Myanmar. Falciparum malaria was endemic in 15 provinces in the 1950s. The malaria control programme in 1955 was successful in eliminating malaria in the central provinces. By 1998, falciparum malaria was endemic only in the two southern most provinces of Yunnan and Hainan. Hainan accounted for 46% of the annual *P. falciparum* cases (Lin et al., 2009, Xiao et al., 2012, CDC, 2012a).

According to WHO, RBM Highlights Report of 2000-2001 approximately 30% of the Central Asian population live in malaria risk areas. In Tajikistan, the disease is endemic in the southern part bordering Afghanistan. However, epidemics have been reported from the central, western and northern regions of the country. Epidemic situations have been assumed in Uzbekistan and are becoming prevalent in Turkmenistan spreading along the western border along Iran. In Turkey about 23% of the population live in high risk malaria regions with approximately 28 million people living in regions at high risk of epidemics (RBM, 2001).

1.9. Malaria in Pakistan

Pakistan is a low malaria endemic country, where malaria is prevalent in areas below an elevation of 5000 feet before 1950 (Bouma, 1996b) but now the limits of malaria prevalence extended to higher altitudes (Map, 2009b) and particularly associated with rice growing regions (Rowland et al., 2002a, Rowland et al., 2002b). The major Anopheline vectors observed in Pakistan are *An. culicifacies* and *An. Stephensi*, with two species of Plasmodium: *P. falciparum* and *P. vivax*. The main features of both vectors are their strong preferences for animals (zoophily), particularly cattle and to rest indoor (endophily). Their preferences to feed on animal blood and their short life span make them poor vectors in theory (Bouma, 1996b). In Pakistan the major malaria vectors are highly zoophilic, and Afghan refugees and local residents prefer to keep their cattle in the courtyard where they sleep in the summer. Therefore conditions for successful passive zooprophylaxis in Pakistan seem highly conducive. However, SOTA & MOGI (1989) mathematical model has predicted that introduction of cattle can lead to increased vector densities as result increased human biting rates, which can lead to greater malaria transmission (Bouma et al., 1995).

Transmission of malaria is seasonal and occurs mainly after the July-August monsoon season (Rowland et al., 2000). There are usually two peaks of *vivax* malaria in a year; one in spring (early April), which may be new transmission or may be relapses from the previous year and the second in summer (September), which is new transmission (Rowland et al., 1997a, Bouma et al., 1996a). Previous studies suggest a single peak of *falciparum* malaria after the summer peak of *P. vivax* (Rowland et al., 1997a, Rowland et al., 1997a). The peak transmission of both species of malaria in Pakistan is suggested to occur in October-November (Rowland et al., 2002 b, Bouma et al., 1996a). The key risk factors to enhance or prolong the transmission season in Pakistan are high rainfall in autumn or above-average temperatures in November-December (Bouma et al., 1996a).

Pakistan is one of the malaria endemic countries in South Asia and major epidemics were recorded in the past, the most devastating outbreak of malaria occurred before the Pakistani independence in 1908 with over 300,000 deaths in an estimated population of 20 million (de Zulueta et al., 1980), while the last epidemic was recorded in the mid-1970s (de Zulueta et al., 1980). Malaria in Pakistan has shown long term

periodic cycles, where many areas are prone to epidemics (Roll back Malaria NWFP Pakistan, Rowland, 1999a). Resurgence of malaria is a growing public health concern for many countries including Pakistan due to migration, deforestation, urbanization and changes in human host's immune suppression.

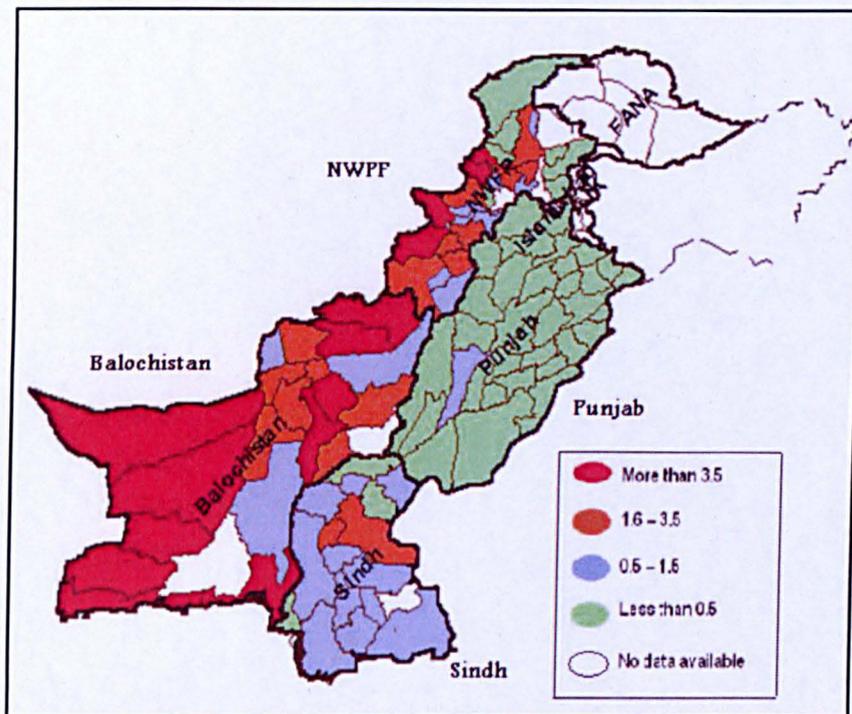


Figure 1.7 Annual Parasitic Incidence (API) of malaria in Pakistan by district (Govt of Pakistan Ministry of Health Web Site, 2006).

The map shows district level API per 1000 population of malaria in Pakistan for the year 2004. The data is passive (clinical). Individual maps of *P. falciparum* and *P. vivax* mated limits are given below.

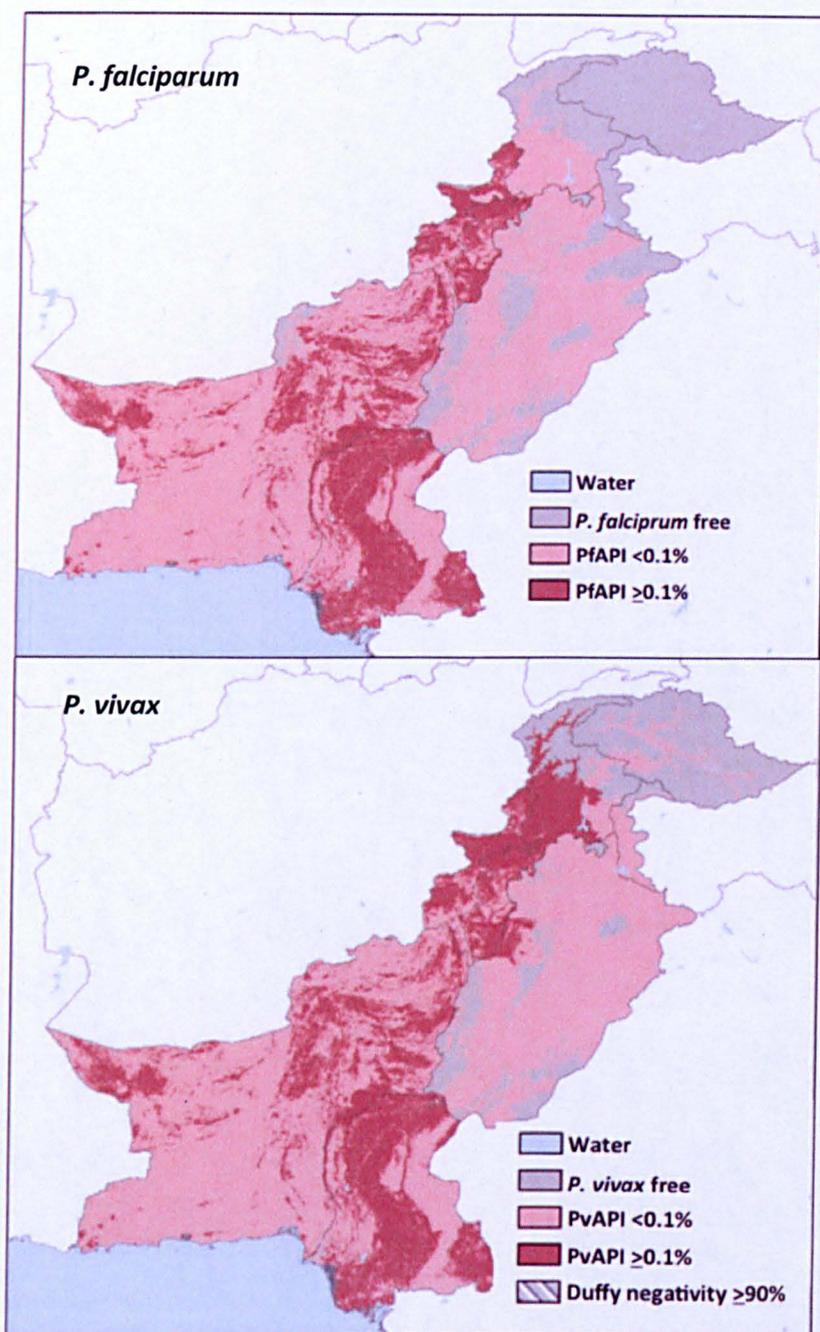


Figure 1.8Es mated limits of *Plasmodium falciparum*(MAP, 2010) and *Plasmodium vivax* malaria (MAP, 2009b)transmission.

In the maps above risk-free areas are shown in light grey. Light pink areas represent unstable malaria transmission. Red coloured areas represent stable malaria transmission. Annual case incidence data over the most recent four years was used. Annual case incidence district level data over the most recent four years was used.

1.9.1. Malaria control in Pakistan

Pakistan initiated a Malaria control programme in 1950 which became an eradication programme in 1960 (Asif, 2008). However, due to epidemics of malaria in the seventies, the Programme strategy was reversed back to malaria control with the programme being decentralized and integrated into the Primary Health Care infrastructure in 1975 in line with WHO strategic policy (Roll back Malaria NWFPPakistan). The program aims to effectively control malaria to improve the health status of the population and had a five year plan to reduce malaria burden 50% by the year 2010 to achieve the WHO global RBM target (Asif, 2008).

According to WHO, although Pakistani malaria control programmes are well established, it is still one of the major health problems of the country, mainly because of the lack of skilled staff, forexample nationwide monitored Rural Health Centres lacked staff to run the Malaria Control programme. In addition, there are funding shortages, lack of health infrastructure, widespread poverty, few qualified doctors and poor data monitoring. Although malaria is the fourth most common medical condition after diarrhoea, ARI [acute respiratory infection] and tuberculosis, its control is hampered by the availability of accurate data at the moment whereby there is a need to strengthen the existing surveillance system (IRIN., February 2006). ITNs/LLINs were adopted in 2008 and distributed free of charge as recommended by WHO. IRS as a malaria control strategy was adopted in 1961; however, DDT is not used for IRS at present due to resistance. IPT (Intermittent Preventive Treatment) for malaria prevention during pregnancy is not in use (WHO, 2012a).

Table 1.4 WHO recommended case management policies in Pakistan adapted from (WHO, 2012a).

Case management	Implementaion (Yes/No)	Year adopted
Patients of all ages should receive diagnostic test	-	-
RDTs used at community level	no	
ACT (Free for all ages in public sector)	yes	2009
Pre-referral treatment with recommended medicines	yes	2009
Oral artemisinin-based monotherapies are not registered	yes	2009

Table 1.5 Pakistan an malarial policy and recommended an malarial drugs (WHO, 2012a).

Antimalarial policy	Medicine	Year adopted
First-line treatment of unconfirmed malaria	Chloroquine	-
First-line treatment of <i>P. falciparum</i>	Artisunate+Sulfadoxine-pyrimethamine	2007
Treatment failure of <i>P. falciparum</i>	Quinine	-
Treatment of severe malaria	Artemether; Artisunate;Quinine	2007
Treatment of <i>P. vivax</i>	Chloroquine +Primaquine(14d)	2007

The recommended malaria treatment for Afghan refugee is standard three-day chloroquine treatment (25 mg/kg) for first episodes and extended five-day treatment(40 mg/kg) for recrudescant infections, based on the assumption that a five-day course would more likely achieve a cure (Haward et al., 2011). To reduce the risk of haemolysis (G6PD deficiency) and enhance adherence to treatment a 5-day course of PQ for vivax malaria is used commonly in South Asia. In Pakistan and India studies have shown that the 5-day PQ course is ineffective at reducing relapse rates and a recent review concludes that 5-day PQ is no better than chloroquine alone at radical cure. 14-day course of PQ is only recommended where the G6PD status of the individual is known (Leslie et al., 2008).

Local vivax malaria treatment policy is to treat with chloroquine alone while falciparum malaria is treated with sulfadoxine-pyrimethamine and artesunate. Patients are more likely to start with self-treatment at home as this allows them to minimize expenditure. In Pakistan, most of the patients in rural areas administer anti-malarial drugs without laboratory confirmation with preference of injectable forms.

1.9.2. Khyber Pakhtunkhawa (KP)

Khyber Pakhtunkhawa (KP) formerly called North West Frontier Province (NWFP) consists of 24 districts; the total population is 17.744 million with an annual growth rate of 2.82% (Census of Pakistan, 1998). Kp is among the areas of the highest latitude where malaria transmission occurs (Bouma, 1996b).

In KP, *falciparum* malaria is particularly unstable and fluctuates markedly from year to year due to climatic variations and malaria outbreaks which have been reported in the past (Bouma, 1996b). *P. falciparum* transmission season starts in the summer monsoon (July) when the temperature and humidity is suitable and remain until the end of the year when the temperature falls below the critical value (December) (Bouma, 1996b). *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, 1996b).

Malaria remains a public health problem in all districts of KP but 2 geographical clusters lie in the northeast (Malakand, Buner, Shangla, and Dir) and the south west (Kohat, Lakki Marwat, Bannu, Tank, Dera Ismail Khan) were previously recorded at high risk of malaria, defined by number of slides confirmed positive by peripheral blood smear, annual parasite incidence (API) and number of cases of *Plasmodium falciparum* (Asif, 2008). Especially small part in the South (e.g D.I.Khan that border Punjab to the South) is notorious for epidemics in the past. In KP more than 50% of the rural health centres have no trained staff to run malaria control programmes (Asif, 2008, Govt of Pakistan Ministry of Health Web Site, 2006). Although, Pakistan and KP had committed to follow the Roll Back Malaria Standards they have been unable to implement these changes due to lack of investment in capacity development and infrastructure (Asif, 2008).

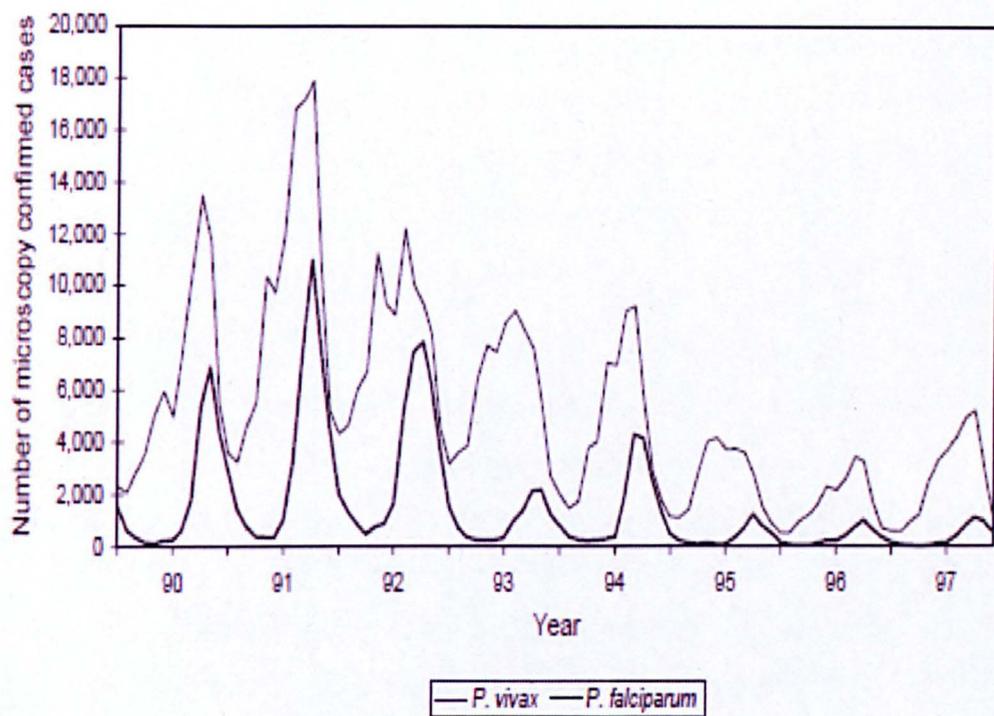


Figure 1.9 Malaria in NWFP recorded by Afghan Refugees Health Programme (ARHP) 1990–1997 (Rowland et al., 2002 b).

1.9.3. Malaria in Afghan Refugee Camps

Migration of refugees from one country to another can affect the distribution and incidence of malaria and may stimulate outbreaks if the new environment is suitable for transmission, e.g refugee camps on the Pakistan-Afghanistan and Thailand-Burma borders (Rowland et al., 2002b, Rowland and Nosten, 2001). About 3 million (nearly one fifth) of the Afghan population migrated to Pakistan in 1978 due to the Russian invasion (Rowland et al., 2002b, Rowland et al., 2002a).

The refugees were settled in 245 camps in KP, 60 in Baluchistan and 12 in Punjab (Suleman, 1988). Most of their shelters were huts and tents, many of which were established in areas known to be capable of supporting mosquito breeding (Suleman, 1988). As the refugees were non-immune at the arrival due to successful malaria control programme in Afghanistan before the Russian war, malaria became a significant health threat on arrival of the refugees to KP (Suleman, 1988). Several epidemics were recorded among refugees in the 1990's. Over 150000 cases were being diagnosed and treated each year by the combined health services of the UNHCR, the government of Pakistan and NGO (Rowland, 1999a, Rowland and Nosten, 2001). The number of cases has declined due to control measures taking effect (Rowland et al., 2002b).

In the early 1990s, after the fall of the Soviet-backed regime about half the refugees were able to return home to safe areas however due to consequent terrorist incidents in the US and the invasion of Afghanistan, the number of Afghans entering Pakistan increased dramatically resulting in a restoration of the former size of the refugee population (Rowland and Nosten, 2001). As health services scale down due to refugees repatriating to Afghanistan, there is a risk that it will re-emerge.

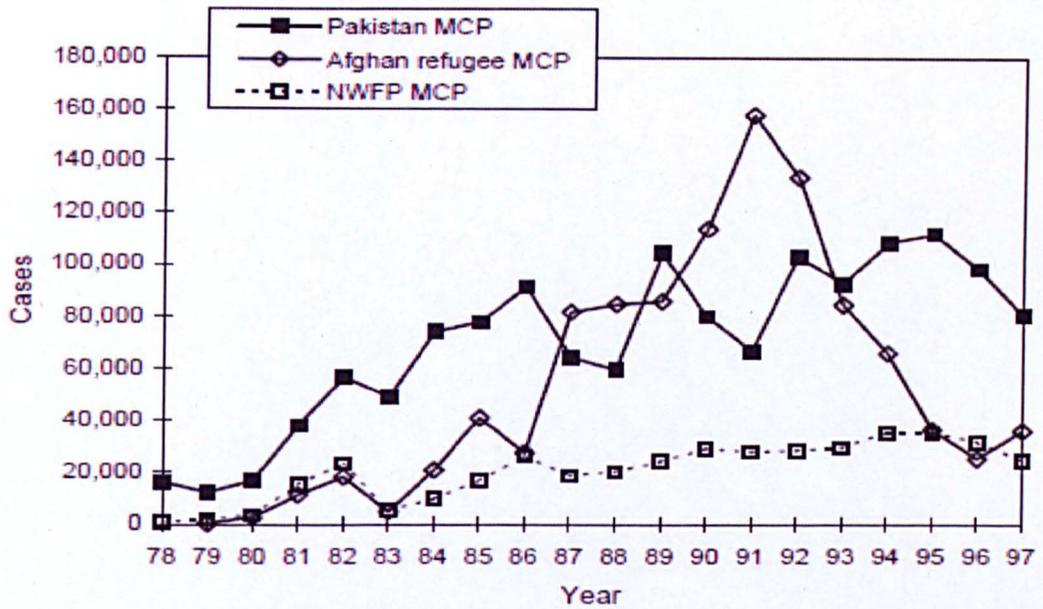


Figure 1.10 Cases of malaria recorded by the malaria control programmes (MCP) of the Pakistan Ministry of Health (for Pakistanis), the NWFP Department of Health (for Pakistanis) and Afghan Refugees Health Programme (ARHP) (NWFP, Punjab & Baluchistan) reproduced from (Rowland et al., 2002 b).

1.9.3.1. Malaria control in KPand in Afghan refugees

In Kp malaria control has relied mainly on the vector control by using insecticide residual spray. From the late 1950s to 1970s 75% wettable powdered DDT was used at 1-2 g/m² often in two rounds/year. Due to wide spread DDT resistance in both vector species reported in the country, malathion (an organophosphate, 50% wettable powder) was introduced as an alternative. Malathion has been the mainstay of malaria control in Pakistan for more than 20 years. It was used at a delivery dosage of 2 g/m² from 1976 to 1977 in double round and then in 1978 a single round was used in July and August. In 1983-1984 limited quantities of fenitrothion (Bouma, 1996b). However, the failure of malathion to reduce malaria substantially in refugee villages of North-West Frontier Province was reported during early 1990s (Rowland et al., 1994).

In 1991 a major malaria epidemic was recorded in refugees and malaria burden increased from 11,200 cases in 1981 to 118,000 cases in 1991 (Rowland et al., 2002 b). However, over the following decade incidence in the refugee camps fell 25% because of control activities, and by 1997 the burden fell to 26,856 cases per year (Rowland et al., 2002 b). The main control measures operated in Afghan refugee camps included, IRS and ITN (Rowland et al., 2002 b, Rowland et al., 2000, Rowland et al., 1997b).

Malaria control in the Afghan refugee villages was supported by a vertical control program implemented by the NGO HealthNet-TPO and funded by UNHCR, which also provides free quality assured microscopy and treatment services for all residents. Due to the contribution of NGOs, increased awareness of malaria as a public health problem and operational research considerable progress has been made in controlling malaria among Afghan refugees but this progress still demands robust and active surveillance tools for monitoring the pre-eliminating process to intensify and coordinate global malaria control, WHO and its partner RBM partnership.

Aims and Objectives

Chapter 2. Aims and objectives

2.1. Aims of the Project

The aim of this study is to generate current information on malaria infection through parasite prevalence and malaria exposure using antimalarial antibody responses in five refugee camps in Khyber Pakhtunkhwa (KP) province of Pakistan formerly called North West Frontier Province (NWFP). The study also aimed to characterise the risk factors for exposure and infection to both *P. falciparum* and *P. vivax* malaria to assist decision-makers in better targeting malaria intervention and control efforts. Additionally by combining GIS data with measures of exposure and infection, the study designed aimed to map potential foci of infection and exposure to malaria to aid malaria control programmes.

2.1.1. Specific objectives

1. To describe age seroprevalence of malarial antibodies for both *P. falciparum* and *P. vivax*.
2. To identify behavioural and residential risk factors for malaria using prevalence of infection and seroprevalence as an outcome variable.
3. To examine spatial distribution of malaria infection and exposure to identify areas of high and low malaria transmission.

2.1.2. Secondary objectives

1. To measure incidence of carriage of *P. falciparum* and *P. vivax* detected by PCR and how that is related to clinical epidemiology and seroepidemiology.
2. To determine the incidence of G6PD deficiency mutations in the study population.

2.2. Results chapters according to objectives

Chapter 4 Assessment of malaria exposure and infection in Afghan refugee camps

Low endemic areas are suitable for malaria elimination but accurate data on the proportion of malaria cases and population at risk are essential for the design and implementation of new interventions. In low endemic areas, the commonly used methods including, EIR, parasite rate and incidence data have been observed to be less sensitive. Therefore, all these three tools, serological markers to assess malaria exposure and RDT and PCR to determine prevalence of infection were used together to get complete information about the prevalence of infection and exposure to malaria in the study area. Since malaria endemicity in study area has been reported very low, it was expected to be difficult to assess malaria transmission intensity and evaluate burden of the disease by using only infection data or exposure data.

Polymerase chain reaction (PCR) was used for more sensitive detection of parasite for assessing prevalence of low-density malarial infection as RDT was expected to miss a substantial proportion of malaria infections due to lack of sensitivity at low parasitemia expected in this low endemic area (Hay et al., 2008, Yekutieli, 1960). It is essential to take into account the extent of the population submicroscopic parasitemia acting as a reservoir for malaria transmission (Okell et al., 2009, Okell et al., 2012, Roper et al., 1996, Schneider et al., 2007, Snounou et al., 1993a). PCR results were also used to examine the effect of parasite carriage on seropositivity and also to assess the relative sensitivity and specificity of RDT. Prevalence of infection measured by PCR and RDT was used to establish present transmission patterns in the area.

Every Afghan refugee camp has its own basic health unit (BHU) that maintains detailed health records (including malaria) of each family within the camp. Recently a significant decrease in malaria prevalence among the refugee camps has been reported from several health facilities (BHU) due to malaria control activities especially the distribution of ITN being supported by International Rescue Committee (IRC) and Health Net International (HNI) (Rowland and Nosten, 2001). It is uncertain whether

these reported changes represent a genuine reduction in disease burden or whether they reflect changing in recording practices and presentation. This apparent decline has had a great impact on the implementation of new malaria control activities so needs to be investigated. This study was designed to provide information of sero-epidemiology of malaria in the camps that could be used to monitor changes in transmission over time and space. So the current data on the malaria burden at population level will help to accurately update malaria situation as routine health facility data (BHU) remain limited due to deficiencies in the data collection and variations in the health services utilization as large percentage of refugees treat malaria at home.

Chapter 5 To identify behavioural and residential risk factors of malaria exposure and infection in the studied camps

The distribution of malaria risk and its burden are unequal within the population (Greenwood, 1989, Woolhouse et al., 1997, Carter et al., 2000), which vary from village to village (Ye et al., 2007, Ramachandran, 2010). Previously malaria transmission has been observed to be dependent on the social and behavioural characteristics of individuals (Moore et al., 2004, Osorio et al., 2004, Joshi et al., 2006), and socio-economic conditions of the household (Ramachandran, 2010). Therefore, it is important to take into account human behaviour, socio cultural and economic context for successful control and elimination of malaria at community level (Williams and Jones, 2004, Opiyo et al., 2007). Therefore, this study also aimed to provide information about socio-economic and behavioural risk factors of malaria, such as travelling outside of camp, seeking malaria treatment, ITN use and IRS and to assess its relationship with infection and exposure to malaria in order to improve prevention and control strategies in the area.

Chapter 6 Spatial distribution of malaria infection and exposure to identify hot spots of malaria within the camp

Heterogeneity in infection and exposure to malaria disease was determined within the camps by measuring the spatial distribution of the disease. To achieve this aim, household level GIS data was combined with infection data and serological responses

to map potential 'hot spots' of high malaria exposure and infection. Geographic information systems (GIS) data integrated with malaria data may provide a quick display of malaria transmission. Malaria mapping has become increasingly common throughout the world over the past decade for assisting malaria control and elimination in malaria-prone countries to provide specific spatial information of the disease (Map, 2013, EMRO, 2013, WHO, 2011a).

Chapter 7 Prevalence and molecular characterization of G6PD deficiency in Afghan refugees in KP, Pakistan

Plasmodium vivax is the dominant species (70-90% of cases) in the study area (Rowland et al., 1994, Rowland, 1999a, Rowland and Nosten, 2001) due to its ability of formation of hypnozoites in the liver and so means that one initial infection can cause up to 10 or more subsequent episodes even after effective treatment for acute episodes (Leslie et al., 2008, Leslie et al., 2004). The liver stage hypnozoites act as a reservoir of the disease that may help the disease to continue its transmission cycle and may reduce the protective efficacy of commonly used preventative measures (insecticide treated bed nets) (Leslie et al., 2010, Leslie et al., 2008).

The radical cure for latent stage vivax malaria is primaquine but the high prevalence of G6PD deficiency and the lack of G6PD testing facilities among the studied population prevent its use. In G6PD deficiency primaquine triggers an acute hemolytic reaction (Bouma, 1995, Ali et al., 2005). Prevalence of G6PD deficiency in Afghans has been observed in several studies ranging from 2 to 10%. Due to occurrence of G6PD deficiency in the study population, the study also focused on the prevalence and its molecular characterization. Since G6PD testing is not readily available, anti-relapse therapy cannot be made widely accessible to most populations at risk of vivax malaria.

Materials and Methods

Chapter 3. Materials and methods

3.1. Study Area

This study was conducted in five Afghan refugee camps in two districts, Mardan and Peshawar of Khyber Pakhtunkhwa (KP) province. Three of the camps; Baghicha (camp 2), Kagan (camp 4), and Jalala camps (camp 3) are located in the Mardan district whereas Adizai (camp 1) is located in Peshawar district and Zangal Patai (camp 5) is located in the Malakand agency (tribal area). Afghan refugees have been resident for more than 30 years in these different camps. The area is classified as malaria endemic and is characterised by seasonal transmission occurring from June to November (Rowland et al., 2002b). The primary malaria vectors are *Anopheles stephensi* and *A. Culicifacies*. Malaria is caused by two species *P. vivax* and *P. falciparum* but predominantly due to *P. vivax* ($\approx 85\%$ - 95% cases) in the studied population (Leslie et al., 2009). The camps are situated in an area which is a well irrigated agricultural plain where sugarcane, wheat and rice are the main crops except for Zangal Patai camp (Table 1.1). House compounds are predominantly constructed from mud and stones separated by a high perimeter wall (Leslie et al., 2009). If animals are owned it is common practice to keep cattle, water buffalos and other domestic animals at night in the owners' compounds. Therefore, almost all sources of mosquito blood meals and indoor resting sites are situated within the camps (Rowland et al., 2001).

3.1.1. Health infrastructure

The province has a developing health system and the refugees most commonly use the Basic Health Units (BHU) run by United Nation High Commissioner for Refugees (UNHCR) and non-governmental organizations (NGO) (Rowland and Nosten, 2001, Rowland et al., 2002 b). These BHUs provide free primary health care services to the population of the camps. Malaria diagnosis is available at BHU level and above (NGOs main offices which are working in Afghan refugee camps), where control program provides free quality-assured microscopy for fever cases diagnosed parasite positive by BHU (Rowland et al., 2002 b). Use of rapid diagnostic tests is infrequent and has not yet been widely implemented, possibly due to high cost. Malaria treatment is free for all

residents of the Afghan refugee camps. Local malaria treatment policy for *P. vivax* is treatment with chloroquine alone with standard 14 day course (Leslie et al., 2008, Leslie et al., 2007, Leslie et al., 2004), while falciparum malaria (now constituting less than 5% of annual cases) is treated with sulfadoxine-pyrimethamine and artesunate (28 days) (Leslie et al., 2009). All BHUs in the selected camps were also included in the study to obtain historical data of malaria cases (appendix 11).

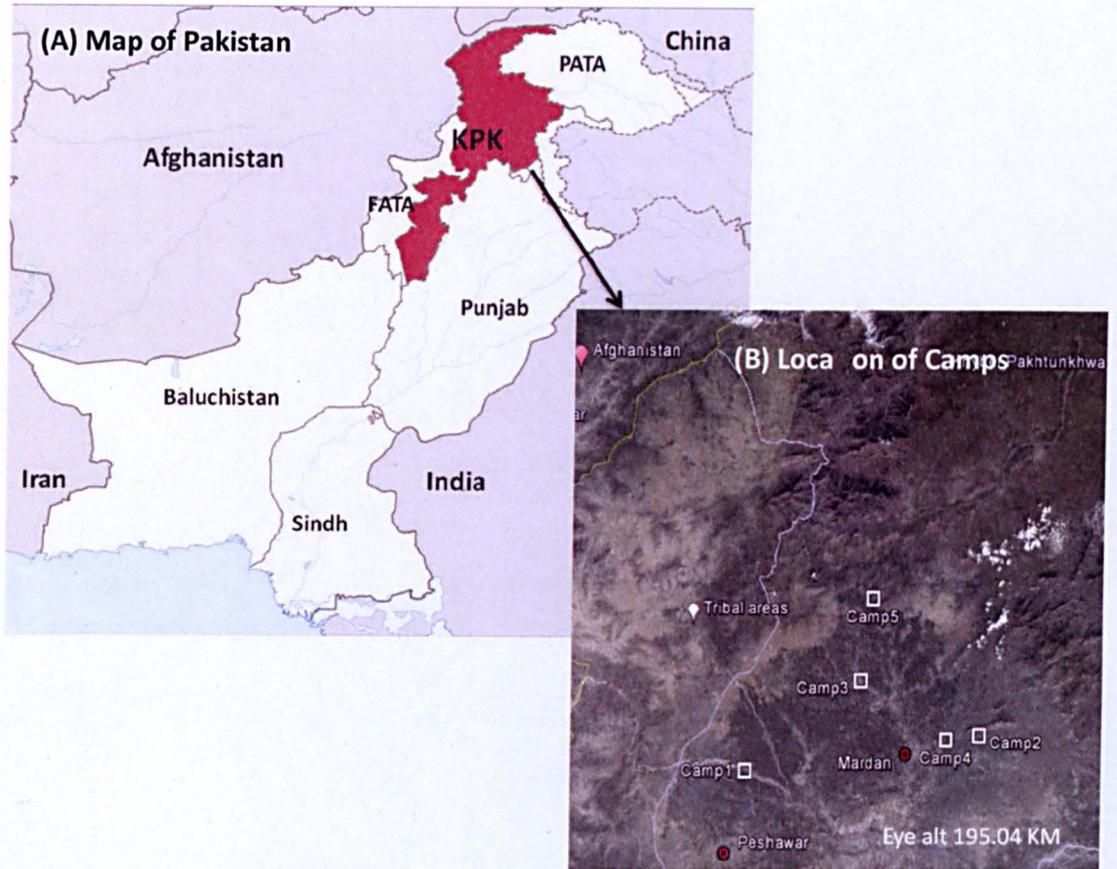


Figure 3.1 Map (A) showing Pakistan its neighbouring countries and KP and map (B) showing location of studied camps in KP.

Map (A) is the map of Pakistan, showing the four provinces (KP, Punjab, Baluchistan and Sindh), provincially administered tribal areas, federally administered tribal areas and neighbouring countries (India, Iran, China and Afghanistan). The highlighted red coloured area is KP bordering Afghanistan. Map (B) showing the geographical position of each studied camp in relation to each other and main towns (cities). The white square shaped symbols are the camps while the red small circles are Mardan (main city of district Mardan) and Peshawar (capital of KP). Adezai camp is labelled as camp 1, Baghicha camp as camp 2, Jalala camp as camp 3, Kagan camp as camp 4 and Zangal patai camp as camp 5.

Table 3.1 Environmental characteristics of the refugee camps in the survey

Camps					
Characteristics	Adezai (camp 1)	Baghicha (camp 2)	Jalala (camp 3)	Kagan (camp 4)	Zangal patai (camp 5)
Average altitude (meters)	314	312	376	312	477
Land type	Sandy and marshy	Marginal and marshy	Sandy area	Soil and good for agriculture	Hilly
Agriculture	Rice, sugarcane and wheat (inside camp)	Rice, sugarcane and wheat (adjoining camp)	Sugarcane and corn (outside camp)	Rice, sugarcane and wheat (adjoining rice irrigation)	Sugarcane and corn (outside camp)
Occupation	Business and labour	Agriculture, jobs and business	Business and keeping sheep and goats (some of them are nomads)	Agriculture, jobs and business	Business and labour
Predominant House construction	Rocks and mud	Bricks and mud	Bricks and mud	Mud	Rocks and mud
Camp sited on/Location with water bodies	Water logged /Along the river	Water logged /Far away from river,	Dry sandy waste land/At a height from river	Water logged /Small water channels for irrigation	Dry rocky waste land or scrub/At a height from water canal
Recent Flooding	2010	No	The river beside the camps was flooded but the houses are on the height were safe	2009	No
Surveyed	During transmission season (August). Half camp before and half after flood	Before transmission season (2 nd week of July before rainy season).	During transmission season (August). After flood	Before transmission season (last week of June)	During transmission season (September). After flood
Likely mosquito breeding sites	Marshes, water pits	Irrigation water and Rice paddy	Irrigation water	Rice paddy and Irrigation water	Streams



Figure 3.2 An open drainage in a street of camp 4



Figure 3.3 Rice field in the vicinity of camp 4

3.2. Study Design

3.2.1. Cross-sectional Survey

A systematic cross-sectional malariometric survey was conducted in each of the five camps, between 24th June and 19th of September 2010. The BHU of each camp maintains good records of malaria history of all households and population size of the camp, where previously each family has a health card issued by BHU. The BHU of each camp was approached to obtain the sketch map of the camp and a recent household list made by BHU polio campaign team.

Selected households for the study were visited by a team of 3 surveyors (already trained and briefed for the survey) from the BHU. In each case the household head was identified and if consent was obtained, questionnaire was administered and a finger prick blood sample was taken from three members of the household, one from each of the three age groups 1-5, 6-20 and >20 years. The household head(s) or primary caretaker(s) was interviewed to assess household wealth indices and about use of anti-malarial measures including the use of ITNs, IRS, coils, sprays, recent treatment of family members with anti-malarial drugs (detailed questionnaire is given in appendix 1).

3.2.2. Sample Size

The desired sample size for this study was calculated based on antibody seroconversion rates by using maximum likelihood estimations. Previously published surveys (Drakeley et al., 2005) were used to derive a model of the dependence of the confidence interval of λ (SCR) on sample size. This model estimates sample sizes by assuming that confidence intervals scale with the number of positive individuals sampled (i.e. it becomes more accurate the greater the number of seropositive individuals identified). It fits a logarithmic function to the measured SD values to calculate sample size as the number of samples that need to be tested (n) to generate sufficient sero-positive samples at different values of SCR and therefore transmission intensity to give the required confidence limits (as % relative standard deviation or %RSD) (Dr P H Corran personal communication). For this study a sample size of 500 individuals per camp was required to define a sero conversion rate (λ) of 0.01 with a

residual standard deviation (RSD) less than 25%. The required sample was 167 compounds assuming 3 individuals per compound (i.e. 500/3), however 200 households were surveyed to allow for refusal or absence at the time of survey.

Table 3.2: Number of samples (n) that need to be tested to generate the required number of seropositive samples, for a given relative standard deviation, at each level of transmission

%RSD desired	No seropositive samples required	λ (yr ⁻¹) (approx. EIR)						
		0.001 (0.002)	0.005 (0.035)	0.01 (0.13)	0.05 (3)	0.1 (11)	0.15 (24)	0.2 (41)
		N						
5	522	18471	5147	3191	1187	802	642	550
10	305	10790	3006	1864	693	469	375	321
15	178	6303	1756	1089	405	274	219	188
20	104	3681	1026	636	237	160	128	110
25	61	2150	599	371	138	93	75	64
30	36	1256	350	217	81	55	44	37

3.2.3. Sampling procedure

3.2.3.1. Selection of Compounds

The pre-numbered list of households prepared by polio campaign team of the BHU was used to select households for the study. The first sampled house was chosen by taking a random number by using a computer generated randomized number list and further, houses were selected systematically in the sequence moving up by one sampling interval each time; for example if number 4 was randomly generated then the 4th, 8th, 12th, 16th etc houses on the list were selected. The same procedure was repeated by moving systematically street by street until the entire camp was covered. Households who refused or who were uncontactable were excluded and not substituted or replaced as this was allowed for in the sample size calculation. The number of people from each age group in each selected household, household size and the number of households in a compound (household from a compound with more than one) were collected in the database to weight the data.

3.2.3.2. Selection of Households: In case more than one households in a compound

Most of the compounds selected comprised of a group of households. In this case all households in a compound were numbered and then one household was randomly selected based on computer generated randomized number list (detailed procedure is given in appendix 17).

The following definition of household was used; A household is a family with one head of the household and sharing one kitchen. In Afghan culture there is a joint family system, in which families of two or more brothers live together in one compound, sharing a kitchen and having one household head (typically the elder brother or their father). The number of households per compound ranged from 1-6 in refugees camps.

3.2.3.3. Sampling within houses

For each camp three randomly selected individuals were chosen from each of the selected households. All members of a household were numbered using computer randomized tables and then one from each of the three age groups 1-5, 6-20 and >21 years were randomly sampled. These numbers have been selected to generate a calculated sample size of at least 500 individuals per camp (detailed procedure is given in appendix 18).

3.2.4. Sample Collection

A finger prick blood sample was taken from all consenting members of the household. Blood samples were collected as three blood spots on filter paper (Whatman, 3MM) which has previously been shown to be a suitable source of antibodies for serological and molecular diagnosis in epidemiological surveys (Corran et al., 2008).

3.2.4.1. Rapid Diagnostic Test (RDT)

All study subjects were screened to determine infection status using CareStart 3-line Pf (HRP II) Pan (pLDH) combo rapid diagnostic test (CareStart™ Malaria RapydTest). 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) (personal communication with author). 5µl of blood sample was taken in the pipette provided by the company and then the blood was released into the sample well. The RDT was left for 20 minutes to develop as suggested by the manufacturer. All malaria positive cases were referred to the nearest health facility for full evaluation and appropriate treatment of their illness.

3.2.5. Sample Storage

Filter paper blood spot (FPBS) samples from the camps were dried and stored in self-sealing plastic bags. Individual bagged FPBS were combined into sets and stored within two further successive plastic bags, the inner most of which contained approximately 3g self-indicating silica desiccant gel. The bags were regularly checked to confirm that the status of the desiccant and was replaced as necessary (Corran et al., 2008). FPBS were stored at 4°C in the Department of Zoology at Peshawar University, Pakistan and

after completion of survey the samples were shipped to the London School of Hygiene and Tropical Medicine and stored at -80°C for long term storage.

3.3. Ethical approval

Ethics approval for the study was granted by both Peshawar University (local Ethics committee) and the LSHTM ethics committee (copies of both ethics clearance are attached in appendices 19 and 20 respectively).

3.4. Laboratory Methods

3.4.1. Serology

3.4.1.1. Selection of antigen

As this study was conducted in a low transmission area, population immune responses were also expected to be low. To detect a low level of exposure, immunogenic blood stage antigens (AMA-1 and MSP-1₁₉) were chosen as antibody responses can be detected and measured following very few infections (Drakeley et al., 2005, Corran et al., 2007, Bousema et al., 2010c, Cook, 2010, Cook et al., 2010). Pre-erythrocytic stage antigens (CSP and LSA) are relatively less immunogenic due to less abundance (or expression) of antigen and a shorter duration of exposure to host immune system (Cook, 2010). Studies have shown that measuring MSP-1₁₉ responses may be more suitable for areas of very high transmission as prevalence of anti-AMA-1 antibodies approaches 100% very early in life. As MSP-1₁₉ is less immunogenic antigen compared to AMA-1, antibody responses may be more informative about malaria exposure in areas of very low transmission (Drakeley et al., 2005, Orlandi-Pradines et al., 2006, Bousema et al., 2010c, Cook, 2010).

It is evident from some studies that MSP-2 is a highly polymorphic antigen (Franks et al., 2003, Hoffmann et al., 2001). Measuring antibody responses against just one variant can therefore lead to an underestimation of seroprevalence rates as other variants circulating in the community of interest will not be detected using ELISA. This makes it a less ideal choice for sero-epidemiological studies (Corran et al., 2007).

3.4.1.2. Production of MSP₁₁₉-GST using E.coli

P. vivax MSP-1₁₉ recombinant antigen was prepared using the reagents, media and culture plates (LB-Amp plates) as detailed in appendix 15. To grow *E.coli*, the surface of the frozen glycerol stock was scraped with a sterile loop and streaked onto a LB-Amp plate. The plate was incubated at 37°C overnight. A single colony was then selected and streaked onto an LB-Amp plate and incubated overnight at 37°C.

Small scale culture: 10ml LB-Amp medium was dispensed into a sterile tube. A single colony was picked off from a LB-Amp plate with a pipette, the tip was ejected into the sterile universal and incubated in a shaking incubator overnight.

Large scale culture production: 500 ml LB-Amp medium was dispensed into each of two sterile flasks (2 litres). 5 ml of small-scale culture was added to each flask and shaken in a shaking incubator at 35 °C for about 6 hours. IPTG stock of 0.5 ml (final concentration 0.1 mM) was added to each culture and was shaken for a further 30 min at 35°C. Flasks were maintained overnight at 4°C. The following day half of the supernatant was discarded and the cells re-suspended by swirling. Each flask was poured into a 500 ml centrifuge tube, which was centrifuged (Sorvall RB50) at 4°C and 5000 rpm for 15 min and the supernatant discarded. The cell pellets were resuspended in PBS and transferred to 50 ml centrifuge tubes. This was washed with PBS and 100 µl was taken as a 'whole cell' sample which was frozen at -20°C.

Cell suspensions were cooled in an ice bath then sonicated for 5 min in 30 sec bursts with 20 sec intervals in between, whilst cooling on ice. 1/20th volume of 20% Triton X-100 was added and mixed for 1h on a rolling mixer at 4°C. 100 µl of sample was taken as 'sonicate', which was frozen at -20°C. The cells were distributed into 2 x 50 ml high-speed sterile centrifuge tubes then spun at 10,000 rpm for 20 min to pellet cell debris. The supernatant was removed into a 50 ml conical graduated centrifuge tube then 100µl of supernatant was taken and frozen. The pellet was re-suspended in PBS and 100µl of sample as 'pellet' was and frozen at -20°C.

Recovery of GST fusion partner by GSH-agarose chromatography

Preparation of GSH-agarose: 3 ml of glutathione-Sepharose 4B was aliquotted into a 50 ml conical centrifuge tube which was made up to 50ml with PBS. This was rotated

on roller stirrer for 2 min and centrifuged at 1000 rpm for 5 minutes. Supernatant was carefully decanted and 5 ml PBS was added per 2 ml of original sepharose stock. This was gently swirled to resuspend.

A nity puri ca on of GST-fusion protein (centrifuge method):

5 ml of washed GSH-sepharose suspension was added to the sonicated supernatant from the previous step. The mixture was incubated on a roller mixer at 4°C for an hour and then centrifuged at 1000 rpm for 5 min. The supernatant obtained was saved and a 100 µl sample kept for SDS PAGE as 'Flow through'. The tube was topped up with PBS, rolled for 2 min and then centrifuged twice with the supernatant discarded each time. The pellet was suspended in an equal volume of PBS then transferred to 15 ml conical centrifuge tube, centrifuged and the supernatant discarded. GSH elution buffer was added to the washed sepharose-GST-fusion protein and rotated for 1 hr. The supernatant was transferred to a bijou and 2.5 ml of GSH elution buffer added and mixed for at least 1 hr. This was centrifuged and added supernatant to first elute and removed an aliquot (100µl) for PAGE. The rest of the eluate was dialysed against PBS at 4°C and frozen at -20°C.

Con rma on of product: SDS PAGE was performed on 10 µl of each aliquot (5µl of crude sonicate and 5ul of pellet), this was stained and the products molecular weight was confirmed (19.GST has M~40 k, GST alone ~29K, single EGF domains ~35K).

3.4.1.3. Titration of Antigen

To assess optimal ELISA coating concentration of the antigen, 600µl of a 1/50 dilution of the antigen was prepared in coating buffer (the preparation procedure for buffers used is detailed in appendix 16). 100µl of coating buffer was added to columns 2-12 and rows 1-4 of an Immulon 4 HBX flat bottom plate (Thermo Scientific), wells A1 to D1 were left empty. 150µl of diluted antigen (1/50 in coating buffer) was added to wells A1 to D1 and then with multichannel pipette 50µl of diluted antigen from column 1 was transferred to column 2 and mixed well. 50µl of diluted antigen was transferred from column 2 to column 3, mixed and transferred to column 4 and so on to column 12. 50µl was discarded from column 12 and the plate was incubated overnight to enable binding of the antigen to the plate.

After overnight incubation, the plate was washed 3 times with PBS/Tween (0.05%), and then 150µl of blocking buffer (1% skimmed milk in PBS/Tween 0.05%) was added to each well and incubated at room temperature for 3 hours. The plate was then washed 3 times and 100µl of 1/1000 dilution of positive control (diluted in blocking buffer) was added to each well and the plate was incubated overnight at 4°C.

The following morning the plate was washed 5 times with PBS/Tween (0.05%) and 50µl of diluted horseradish peroxidase-conjugated rabbit anti-human IgG (Dako #P0214), diluted 1/5000 in PBST, was added to each well, then the plate was incubated for 3 hours at room temperature. After washing a further 5 times, 100µl of OPD (SigmaFast OPD tablets, Sigma) substrate solution was added and left at room temperature in the dark for 15 minutes for the assay to develop. The reaction was stopped by adding 25µl per well of 2M H₂SO₄ and the plate was read at a wavelength of 492nm in ELISA reader to get raw data (ODs).

The data was transferred to an Excel spreadsheet, and then ODs were plotted against the antigen dilution on a log scale. A coating concentration was chosen, which corresponded to a dilution just below where the curve was saturated. In the graph below, the 1/4000 dilution was chosen for this antigen, as this corresponds to a concentration where the entire plate surface is coated with antigen and no more will bind (Figure 3.2).

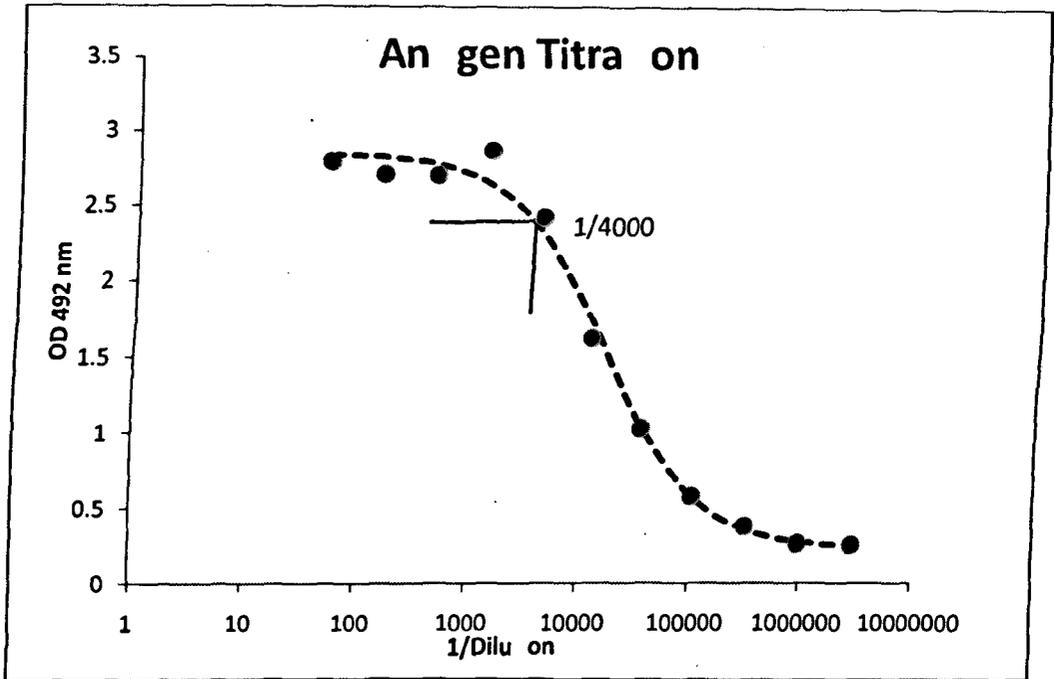


Figure 3.4 Graph demonstra ng the process for the selec on of suitable coa ng concentra on of an gen, which corresponds to a dilu on just below where the curve saturates

3.4.1.4. Reconstitution of Dried Bloodspots for ELISA

Antibodies were eluted from FPBS using methods previously described (Corran et al., 2008). Briefly, using the leather hole punch, a bloodspot disc of 3.5mm was cut from the middle of the filter paper sample. The bloodspot was placed into a well of a 96 deep well plate (Costar 0.5ml V well bottom assay block) using tweezers, and the sample identity number was recorded (ID) on the plate plan. 280 μ l of the Reconstitution buffer (sodium azide 1g per 1 litre PBS/Tween) was added to each well. The assumption is that a 3.5mm disc holds ~2.8 μ l of whole blood which constitutes a 1:100 dilution of blood equating to a 1:200 dilution of sera, assuming a haematocrit content of ~50%.

Table 3.3 Filter paper reconstitution based on size of circular spot

Diameter (mm)	Amount of blood (μ l)	Volume to reconstitute
1.0	0.2	20
1.5	0.5	50
2.0	0.9	90
2.5	1.4	140
3.0	2.1	200
3.5	2.8	280
4.0	3.7	360
4.5	4.7	460
5.0	5.8	570
5.5	7.0	690
6.0	8.3	820

Consequently, the deep-well plates were sealed with a mat and placed on a plate shaker overnight rocking. The following day if samples were successfully eluted, the filter paper circles appear white and the solution appears pink/red. Samples where the circle was still red and the solution pale were deemed as not eluted and were rejected. Plates were sealed and stored at 4°C for up to 1 month or at -20°C for long-term storage.

3.4.1.5. ELISA

ELISA was used to detect specific antibodies against *P. vivax* and *P. falciparum* malaria to estimate exposure and transmission intensity in the study area. The ELISA protocol was run over a 3 day period to allow the testing of a large numbers of samples during each run. Details of the preparation of specific reagents are given in appendix 16.

After identification of the optimal antigen concentration 96 well ELISA plates (Immulon 4) were marked out and plate template detailing sample locations were recorded in a laboratory record book. Antigen was diluted in coating buffer to the correct concentration (as determined by the titration), taking into account the number of plates that requiring coating. Then 50µl of diluted antigen was added to all wells of the plates for overnight incubation at 4°C.

Next morning ELISA plates were washed three times in PBS/Tween wash solution and 150µl of blocking solution was added to each well. Plates were subsequently incubated at room temperature for three hours. After incubation, plates were washed three times in PBS/Tween and 40 µl of blocking solution was added to each well, followed by 10 µl of sample to each well into duplicate. A serial dilution of a positive control sample (pooled hyper-immune serum from Tanzania) was included on each plate along with 4 wells as blanks, which contained no sample. Plates were then incubated overnight at 4°C.

After overnight incubation plates were washed five times in PBS/Tween and 50µl of conjugate solution (horseradish peroxidase-conjugated rabbit anti-human IgG, Dako #P0214) diluted to 1/5000 in PBS/Tween, was added to each well. The plates were incubated for three hours at room temperature and then washed five times in PBS/Tween. 100µl per of OPD (SigmaFast OPD tablets, Sigma) substrate solution was added to each well and left at room temperature in the dark for 15 minutes for the assay to develop. The reaction was stopped by adding 25µl of 2M H₂SO₄ per well. Plates were read at 492nm in ELISA reader and the raw data was saved.

3.4.1.5.1. Calculating serial dilutions for control sera

Rather than prepare controls separately, a stock of positive controls (a pool of serum from adults living in a hyperendemic region) that was enough for all the ELISA plates was made. This reduces the variability between plates. Serial dilutions dropping fourfold were prepared as follows: 1/10, 1/40, 1/160, 1/640, 1/2560, 1/10240.

3.4.2. Molecular Diagnosis (PCR)

PCR was used to identify low density parasite infections for the assessment of infection and to examine the effect of parasite carriage on seroprevalence. From four camps including camp 1, 2, 4 and 5 a sub set of samples (filter paper blood spots), including all RDT positives and randomly selected 120 RDT negative samples, were assayed by PCR (Snounou et al., 1993a). The selection was carried out by randomly picking a sample (filter paper blood spot) from a well-shuffled bag containing all samples of a camp. Except for camp3 in which all samples (505) were subjected to PCR analysis.

3.4.2.1. DNA extraction from filter paper blood spots using Chelex

A 5 mm disc was cut of each FPBS using a hole punch and placed in a deep 96-well plate. Three +ve controls were added in the last three wells and to each row one negative control was added which was distributed across the deep-well plate. Then 1 ml of freshly made 0.5% saponin (Sigma) was added in 1X PBS to completely wet the filter paper and was incubated at 37°C overnight. Saponin causes haemoglobin to be released into the PBS solution and leaves host DNA (including parasite DNA) on the filter paper.

The plate was centrifuged briefly for 2 min at 4000 rpm and saponin solution and debris were removed using suction pump (using a new tip for each of the well) leaving only filter paper. Then 1ml fresh PBS (1X, autoclaved) was added and centrifuged for 2 minutes. Liquid and debris were removed again. The washing step was repeated with 1X PBS until there was no visible haemoglobin left on the sample. Next 150µl of 6% chelex suspension in nuclease-free water was added to each sample followed by adding 100µl nuclease-free water.

The plate was covered with foil, heat sealed and boiled for 30 minutes in water bath set at 100°C. The plate was centrifuged for 2 min to spin down chelex and remaining

filter paper. 100µl of DNA supernatant was taken and added into a fresh, sterile PCR plate. The PCR plate (DNA) was stored at -20°C. The Chelex beads and filter paper were discarded.

3.4.2.2. Diagnostic PCR for identification of Plasmodium species

An optimised diagnostic nested PCR by Snounou and colleagues (Snounou et al., 1993a) which targets the small subunit ribosomal RNA gene (ssrRNA) was used to detect *P. falciparum* and *P. vivax* in samples.

Species identification was a 3 step process; firstly a set of genus specific primers were used in nest1 (N1) reaction. The N1 product was used in two separate N2 reactions using sets of species-specific primers, one for *P. vivax* and the other for *P. falciparum*. The primers are detailed in Table 3.4. Positive and negative controls were used in all PCR steps for quality control.

Table 3.4 Genus and species specific primers for both *P. falciparum* and *P. vivax*

Purpose	Forward	Reverse
Genus specific	5' TTAAAATTGTTGCAGTTAAAACG 3'	5'CYTGTGTTGCCTTAAACTTC 3'
<i>P. vivax</i> specific	5'TTAAACTGGTTTGGGAAAACCAAATA TATT 3'	5'ACACAATAGACTCAATCATGACTACCC GTC 3'
<i>P.falciparum</i> specific	5'CGCTTCTAGCTTAATCCACATAACTGA TAC 3'	5'ACTTCCAAGCCGAAGCAAAGAAAGTC CTTA 3'

3.4.2.2.1. Nest 1 Reaction

The Nest 1 PCR mix and cycling conditions were as follows

PCR Mix

Working concentration	Final concentration	Reaction volume 20 μ L
dH ₂ O	-	10.5
10XNH ₄ SO ₄ buffer	1X	2
50 mM MgCl ₂	2 mM	0.8
10 mM dNTPs	250 μ M	0.5
5 μ M Primer mix (genus specific)	250 nM	1
Taq DNA polymerase	1 μ l	0.2
DNA	-	5

Cycling conditions

Temp ($^{\circ}$ C)	Time (min.)	Cycles
95	5	1
58	2	25 cycles
72	2	
94	1	
58	2	1
72	5	
25	10	1

3.4.2.2.2. Nest 2 Reaction

Same PCR mixes and cycling conditions were used for both the N2 PCRs.

PCR Mix

Working concentration	Final concentration	Quantity per 20 μ L
dH ₂ O	-	14.5
10XNH ₄ SO ₄ buffer	1X	2
50 mM MgCl ₂	2 mM	0.8
10 mM dNTPs	250 μ M	0.5
5 μ M Primer mix (<i>P. falciparum</i> or <i>P. vivax</i> specific)	250 nM	1
Taq:	1 μ l	0.2
DNA (N1 product)	-	1

Cycling conditions

Temp (°C)	Time (min.)	Cycles
95	5	1
58	1	30 cycles
72	1	
94	1	
58	1	1
72	5	
25	10	1

8ul of the nest2 product was separated on 2% agarose gel ran at 90v for 60minutes. *Plasmodium falciparum* produces 205bp PCR product and for *Plasmodium vivax* the PCR product size produced is 120bp (Figure 3.3).

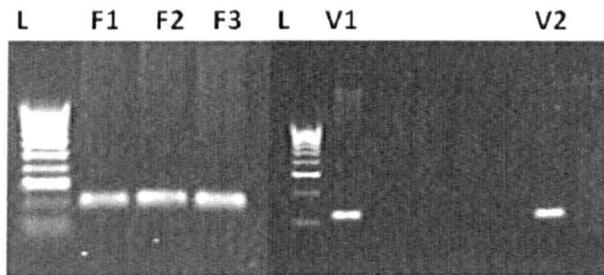


Figure 3.5 Gel photograph showing the nest 2 PCR product of *P. falciparum* and *P. vivax*.

Key to figure 3.3: L=Hyperladder IV (DNA ladder), F1-F3=*P. falciparum* PCR products, V1-V2=*P. vivax* PCR products.

3.4.2.3. Identification of mutations associated with G6PD deficiency

An optimised PCR RFLP (restriction fragment length polymorphism) assay by (Bushra 2009) was used for the detection of G6PD 563C-T [type II]/ 188 Ser→Phe mutations in Jalala camp.

3.4.2.3.1. G6PD PCR

DNA obtained through chelex extraction described above was used as a template in a PCR reaction. Negative controls were added in each set of PCR for quality control.

Primers used

5' ACTCCCCGAAGAGGGGTTCAAGG 3'

5' CCAGCCTCCCAGGAGAGAGGAAG 3'

PCR Mix: The total reaction volume was 20ul.

Working concentration	Quantity per 25 µl
dH ₂ O	10.6/11.1
10XNH ₄ SO ₄ buffer	2.5
50 mM MgCl ₂	1
10 mM dNTPs	2.5
10 µM Primer	1.5/1.5 OR 1/1
Taq	0.4
DNA	5

Cycling conditions

Temp (°C)	Time (min.)	Cycles
95	2	38 cycles
94	1	
67	30s	
72	40s	
72	5	

The product was run on a 1.5% agarose gel at 100v for 60mins.

3.4.2.3.2. Sequencing G6PD PCR product

Prior to restriction of the samples, five G6PD products were sequenced to ensure the position of restriction sites for MbolI (restriction site is 563C-T). The sequencing protocol was as follows:

PCR products were treated with Exonuclease I (ExoI) and Alkaline Phosphatase (AP) (Fermentas) using the following 10ul mix per sample.

Working concentration	Quantity per 10 μ l
dH ₂ O	3.35
10X Alkaline Phosphatase dilution buffer	0.5
Exonuclease I (20U/ μ l)	0.15
Alkaline Phosphatase (1U/ μ l)	1.0
PCR product	5 μ l

The mix was incubated in PCR machine for 60 minutes at 37°C followed by 72°C for 15 minutes.

Sequencing reaction

The BigDye V3.1 manufacturer's protocol (AB) was followed to set up the sequencing reactions. Two sequencing reactions were set up per sample; one with the forward primer and the other with the reverse primer. The reaction mix comprised of:

Working concentration	Quantity per 10 μ l
Nuclease free H ₂ O	6.25
BigDye V3.1	0.5
5x sequencing buffer	1.75
10 mM primer	1
DNA from enzymatic clean up reaction	0.5

Cycling conditions were as follows,

Temp (°C)	Time (min.)	Cycles
96	1	
96	30s	25 cycles
50	30s	
60	4	
4	Hold until purification	

BigDye reac on cleanup

90ul of Ethanol/Sodium acetate mixture was prepared per sample. The mixture composition was as follows.

Working concentra on	Quan ty per 90 µl
Nuclease free H2O	24.5
3M NaOAc (pH 5.2)	3
Absolute ethanol	62.5

For the final clean-up 90µl of the Ethanol/Sodium acetate mixture was added into each 10µl reaction followed by brief vortexing. The tubes were then left at -20°C for 20mins for the extension products to precipitate, after which they were centrifuged at 3000g/4000rpm for 30 minutes. The supernatant was removed by pipetting. 150µl of 70% ice-cold ethanol was added to each pellet and the tubes were centrifuged again at 3000g/4000rpm for 10mins. The supernatant was removed again by pipetting. The tubes were left to dry in dark for 10-30 mins after which 10µl Hi-Di formamide was added to re-suspend the pellet. The tubes were finally submitted for sequencing.

3.4.2.3.3. Restriction of the G6PD PCR product

Once the restriction sites and fragment sizes were established through sequencing, the G6PD product from rest of the samples were subjected to digestion by restriction enzyme *MbolI* (Fermentas kit) that targeted the 563C-T mutation sites in the sequence.

MbolI

Cutting at

5'... G A A G A (N)8↓...3'

3'... C T T C T (N)7↑...5'

The digestion mixture was as follows,

Reagent	Quantity per 30μl
G6PD PCR product	14.5
Nuclease-free H ₂ O	11.3
<i>MbolI</i> enzyme	1
BSA	0.2
10X Buffer B	3

The mixture was incubated at 37°C for 5 hours. The digested product was then run on 2.5% agarose gel at 90v for 120mins.

3.5. Data management and analysis

All data from the cross-sectional survey was collected on paper questionnaires, from which data was entered into the access sheets. Data was analyzed by using Stata12 statistical software. As earlier mentioned, only a sub set of samples were selected to be assayed by PCR, there were expected to be chances of overestimation of infection prevalence or biased results detected by PCR, because all the RDT positive samples were included to be assayed by PCR. Therefore to assign measures of accuracy to sample estimates and to control and check the stability of the results, Bootstrap statistical method was used to estimate the prevalence of infection detected by PCR. The basic idea of bootstrapping is to make conclusion about a population from sample data (sample -> population) by resampling from the sample data.

Modelling of seroprevalence in order to derive measures of transmission intensity and identify changes in transmission among camps was carried out in Stata12, used within the department (Corran et al., 2007). Principle components analysis to evaluate socioeconomic status, logistic regression was used to analyse of risk factors for both *P. falciparum* and *P. vivax*. For spatial analysis to detect hot spots of malaria within the camps SaTScan and ArcGIS softwares were used. The specific statistical tests used are described in detail in the specific chapters where they were used.

3.5.1. Serological data

3.5.1.1. Cleaning and interpretation of ELISA results/data

The raw data (ASCII like PRN files) from the ELISA reader was imported to MS Excel for data cleaning and manipulation. Duplicate OD results differing by a factor of 0.2 were not used in the analysis. For each ELISA plate a titration curve was fitted to the ODs obtained for the standard plasma dilutions by least squares minimisation using a three variable sigmoid model and the solver addin in Excel (Microsoft). Averaged sample OD values were converted to units/ml using the fitted curve, assuming an arbitrary value of 1000 Units/ml of antibody against each antigen in the standard pool.

The concentration (arbitrary units) or magnitude of antibody response is represented as a titre using the following formula:

$$\text{Titre} = \text{dilution} / [\text{maximum OD} / (\text{OD test serum} - \text{minimum OD}) - 1]$$

Standard curves were plotted and normalised on the same graph to assess the difference between them. A plate was repeated if the control curve was considered invalid. Conversion of OD to titre occasionally resulted in negative titres (e.g. when the blank well value was high than the sample OD), these values were adjusted to 0.0001 and retained in the analysis to allow for a continuous antibody concentration. The titre values were skewed and were log transformed.

3.5.1.2. Cut off point calculation for sero-prevalence determination

An important definition in sero-epidemiology is defining a threshold above which a sample is considered sero-positive. There are a variety of different methods which can result in different interpretation of the same data. The standard way to define cut off between sero-positive and sero-negative is to use serum of people who are naive or non-exposed to a pathogen as a representation of sero-negative population. Based on the normal distribution of their antibodies a cut off for differentiation between sero-positivity and sero-negativity is calculated. However, in the case of malaria endemic areas it will be hard to find directly comparable non-exposed subjects, so naive individuals are usually chosen from different settings (i.e. Europeans). However, this approach has the disadvantage that these individuals are of different ethnicity and exposed to completely different environment and infections which may affect generation of immunoglobulin. Also there may be seen nonspecific binding to plasmodium antigens due to differential exposure to other infectious agents. Other factors that also have an impact on immune responses are host genetics (Farouk et al., 2005, Modiano et al., 1996), behaviour and culture such as diet and nutritional status (Grimble, 2001).

In this study serological data were fitted to a mixture model to measure appropriate cut off to define sero-positivity. This method is already used in different sero-epidemiological studies of malaria (Bousema et al., 2010c, Cook et al., 2011, Cook et al., 2010, Corran et al., 2007). In this model it is assumed that the antibody OD results comprise a mixture of two (or more) partially overlapping normal (Gaussian)

distributions. The samples are assumed to represent both a narrow negative distribution and a broader distribution of positive OD's from the population of interest. The distribution of sero-negative subjects is used to define cut off for seroprevalence negativity. The proportions and the parameters in each group were estimated by fitting the model using maximum likelihood methods.

If there are n measurements from y_1, \dots, y_n then the likelihood to be maximised

$$L(\mu_1, \sigma_1, \mu_2, \sigma_2, \rho) = \prod_{i=1}^n [\rho \phi(y_i, \mu_1, \sigma_1) + (1 - \rho) \phi(y_i, \mu_2, \sigma_2)]$$

Where $\phi(y, \mu, \sigma)$ represent the normal probability function for an outcome y with mean μ and standard deviation σ . Where μ_1, σ_1 and ρ are mean, standard deviation and proportion respectively in the lower mean normalised ODs group, while μ_2, σ_2 , are the mean and standard deviation in the other group. Then the cut off for sero-positivity is equal to $\mu_1 + 3\sigma_1$ (Cook, 2010). The mixture model was run on normalised ODs of each antigen in STATA. A separate cutoff value was generated for each antigen (MSP-119 and AMA-1) for each species (*P. vivax* and *P. falciparum*).

Antigen	Cutoff
PfAMA-1	0.07
PfMSP-1 ₁₉	0.13
PvAMA-1	0.43
PvMSP-1 ₁₉	0.09

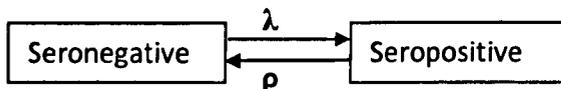
3.5.1.3. Seroprevalence

The normalized ODs were used to produce a cut-off value above which samples were considered antibody positive (Cook, 2010, Bousema et al., 2010c, Corran et al., 2007). Antibody responses were combined by species to determine the presence of any reactivity against *P. falciparum* or *P. vivax* to enhance the assay sensitivity as the overall prevalence of antibody was low (Bousema et al., 2010c). Other serological studies which used a similar model include (Cook et al., 2011, Cook et al., 2010, Cook, 2010, Bousema et al., 2010c, Corran et al., 2007, Drakeley et al., 2005)

Seroconversion rate and modelling age specific antibody prevalences

The seroconversion rate (SCR) which is the rate at which a seronegative individual becomes seropositive in a year was generated by fitting a simple reversible catalytic model to the measured seroprevalence by age in years by using maximum-likelihood methods (Corran et al., 2007, Bousema et al., 2010c, Cook, 2010).

Reversible catalytic prevalence model



λ represents SCR and ρ is the rate of reversion of seropositive individual to seronegative in a year.

The equation for the model is represented by using maximum-likelihood methods

$$P_t = \frac{\lambda}{\lambda + \rho} (1 - \exp(-(\lambda + \rho)t)) \text{-----equation 1.}$$

Where P_t is the proportion of individuals who are seropositive at time t , λ is the annual seroconversion rate which is village specific, and ρ annual seroreversion rate which is area specific. Both seroconversion (λ) and seroreversion rates (ρ) were calculated by fitting the model. The value ρ (seroreversion rate) gives a measure of the persistence of the antibody response. Estimates of ρ will be more reliable when P is large (i.e. for older age groups and for higher transmission locations), and least easy to determine when P is small (i.e. in areas of low transmission) (Corran et al., 2007).

The best fit was obtained using a common value of $\rho(\text{Rho}) = 0.0173$ (0.0135–0.0215) (95% confidence interval by likelihood ratio test) for all camps (single constraint Rho for all camps). A previous study in Tanzania also used one constrained ρ (Rho) for all study sites to measure transmission intensity of malaria by using age seroprevalence data. The authors found that the quality of fit for a single constrained value of ρ was not significantly improved by the use of individual values of Rho estimated for each location, which suggested that ρ might be mostly independent of transmission intensity (Corran et al., 2007).

Chapter Four

Assessment of malaria exposure and infection in Afghan refugee camps

Chapter 4. Assessment of malaria exposure and infection in Afghan refugee camps

4.1. Introduction

The aim of this chapter was to generate current information on malaria infection through parasite prevalence and malaria exposure using antimalarial antibody responses in residents of the study area. A secondary objective was to validate the usefulness of serology, PCR and RDT as tools to monitor malaria endemicity. The study also aimed to provide insight into the nature and role of antimalarial immunity in the target population. Accurate data on the proportion of malaria cases and population at risk are essential for the design and implementation of new interventions. Additional information on the population at risk will help malaria control activities and guide research, as very little data are available regarding infection and exposure to malaria in this particular study area.

As discussed in the main introduction, malaria in Pakistan is seasonal with the highest transmission recorded after monsoon. *P. vivax* is the predominant species which contributes $\approx 85\%$ of the malaria cases (Rowland et al., 2001). In KP falciparum malaria transmission is particularly unstable, fluctuating markedly from year to year due to climatic variations (Bouma et al., 1996a). These have been linked to small outbreaks of *P. falciparum* in the region reported in the past (Bouma et al., 1996a).

KP is characterized as low malaria endemic area with unstable and highly seasonal transmission (epidemic prone) and reported parasite prevalence is $<10\%$ (Kaleem and Jan, 1993, Bano and Mufti, 1980, Bouma et al., 1996a, Rowland et al., 1997a, Muhammad and Hussain, 2003, Awan et al., 2012), which may be suitable for malaria elimination. However, to achieve this accurate data of malaria transmission intensity and burden of the disease of the study area are required. Prior to implementation of any control or elimination programme it is essential to assess the previous malaria situation and to determine the impact of previous control prevention strategies if any.

Entomologic inoculation rate (EIR) is the direct and gold standard measure of transmission intensity. However it has limitations: it is difficult to determine when the number of infected mosquitoes is expected to be very low in low endemic areas and may not reflect the spatial heterogeneity in mosquito densities (Oesterholt et al., 2006, Smith et al., 1995, Drakeley et al., 2003). EIR can be useful measure of malaria transmission intensity in hyper endemic areas but it will be expensive and time consuming in low endemic areas and will demand long-term intensive sampling.

The other most commonly used methods to measure malaria infection are microscopy and rapid diagnostic tests (RDT). But both of these measures are less useful at low levels of parasitaemia due to lack of sensitivity in low endemic areas (Hay et al., 2008, Yekutieli, 1960). They therefore can miss a substantial number samples with low-density parasitemia, which can play an important role as an infectious reservoir (Okell et al., 2009). However, many studies reported that methods based on the Polymerase Chain Reaction (PCR) are more sensitive than microscopy or RDT and its use was suggested for assessing prevalence of low-density malarial infection (Okell et al., 2012, Okell et al., 2009, Roper et al., 1996, Schneider et al., 2007, Snounou et al., 1993a).

An alternative method to measure malaria transmission intensity and past trends in malaria transmission intensity (MTI) is the prevalence of antibodies against malaria parasites (Corran et al., 2007, Corran et al., 2008, Drakeley et al., 2005). This method was previously used in different regions to compare the level of malaria transmission intensity (Drakeley et al., 2005, Corran et al., 2007) and especially useful in areas of low endemicity (Bousema et al., 2010c). Serologic markers to measure exposure to malaria were suggested to be advantageous, especially when the prevalence of infection and infected mosquitoes is low. Because antimalarial antibodies are thought to persist after infections have cleared, antibody seroconversion rates are considered to be less vulnerable to seasonal fluctuations on the timescale of the lifespan of vector or the half-life of discrete parasite infection (Drakeley et al., 2005, Corran et al., 2007).

Since the malaria endemicity in the study area is recorded as very low, it was expected to be difficult to assess malaria transmission intensity and evaluate burden of the disease by using only infection data or exposure data. Therefore all three tools were combined to get the maximum information about the prevalence of infection and

exposure to malaria in the study area. In this section I used RDT/PCR to determine prevalence of infection and serologic markers of exposure to assess exposure to *P. falciparum* and *P. vivax* in different Afghan refugee camps. Parasite positive study participants (detected by RDT or PCR) would be expected more likely to be antibody positive to either antigen or parasites in general.

So the current data on the malaria burden at population level will help to accurately update malaria situation as routine health facility data (BHU) remain limited due to deficiencies in the data collection and variations in the health services utilization as a large percentage of possible malaria cases treat malaria at home. This additional information on the population at risk will help malaria control activities and guide research in the study area.

4.2. Material and Methods

4.2.1. ELISA to detect antibodies to specific malaria proteins

All dried blood spot samples were tested to measure antibody responses (serological markers of malaria infection exposure). Blood spots were reconstituted and ELISA was carried out as described in detail in Materials and Methods (Chapter 3). Briefly, antigens specific for *P. falciparum* and *P. vivax* were coated overnight at 4°C. ELISA plates were washed in PBS and blocked with 1% skim milk powder in PBS/Tween for 3 hours. After further washing, positive controls (a pool of hyper immune plasma) and samples were added in duplicate wells on each plate and incubated overnight. The following day plates were washed 5 times and incubated with horseradish peroxidase conjugated rabbit anti-human immunoglobulin G for 3 hours at room temperature. After further washing, SigmaFast *o*-phenylenediamine dihydrochloride substrate solution was used to develop plates for 20 min. 2 mol/L H₂SO₄ (5 µl/well) was used to stop the reactions and plates were read at 492 nm immediately. Serum was used at 1:1000 dilutions of original.

4.2.2. Molecular Diagnosis

PCR was performed to identify low-density parasite infection for both species on 120 randomly selected RDT negative samples plus all RDT positive samples in four of the five camps including camps 1, 2, 4 and 5 but in camp 3 all the samples (505) were assayed using a malaria specific PCR (Snounou et al., 1993a). These results were used to examine the effect of parasite carriage on seropositivity, assess the relative sensitivity and specificity of RDT and to see sub-microscopic parasite carriage in this population. The method is described in detail in chapter 3.

4.2.3. Statistical analysis

All analysis was conducted in STATA version 12 (Stata Corp LP, College Station, TX, USA) using the survey command (svy) with camp as a stratum and household as the primary sampling unit. Maximum-likelihood methods were used to estimate seroconversion rates (SCR) from age seroprevalence curves by fitting a reversible catalytic model to the measured seroprevalence by age in years. To check for most recent changes in transmission, seroprevalence in individuals with age equal to or less than five years and titres of antibodies which were suggested to be sensitive than seroprevalence were used (Cook, 2010). To test the association between seroconversion rate (SCR) and parasite rate (PR) scatter plots were examined and correlation coefficients were calculated. As mentioned above, only a sub set of samples was selected for assay by PCR: because all the RDT positive samples were included, there was a risk of overestimation bias of infection prevalence. Therefore to assign measures of accuracy to sample estimates and to control and check the stability of the results, Bootstrap statistical methods were used to estimate the prevalence of infection detected by PCR. The basic idea of bootstrapping is to draw conclusions about a population from sample data (sample-> population) by resampling from the sample data.

4.3. Results

Cross sectional surveys were successfully completed in 5 camps with a total of 2522 individuals surveyed. Prior to the surveys demographic data were collected from each camp (Table 4.1). Camp population size ranged from 3790 to 12000 approximately and there were no major differences in demographic composition.

The average family size ranged from 7.9-10.3 and 52-60 % of sample collected were female in the sample. From each selected household three blood sample collected on filter paper from each of the age group mentioned earlier in materials and methods chapter, so approximately 33% individuals were sampled from each of the three age groups. The questionnaire database was matched with all serology data except for 10(0.4%) individuals who had no ELISA data for both *P. falciparum* and *P. vivax*. The missing ELISA results were dropped, as duplicates were not within the specified range of each other as mentioned in main Materials and Methods (Chapter 3).

Table 4.1 Demographic breakdown of study participants by each camp

Camp					
	Adezai (1)	Baghicha (2)	Jalala (3)	Kagan (4)	Zangal patai (5)
Total population (approximate)	6011	10879	12000	3790	8178
Total number of houses per camp (approximate)	411	620	755	401	590
Number of households sampled	169	169	169	166	169
Sample size per camp	507	507	505	496	507
Average family size	8.9	8.4	7.9	8.3	10.3
Family size range	3-22	1-14	2-17	2-15	3-25
Sex(%)					
Female	60	60	65	58	52
Number of individuals selected from each age group/total No. of individuals in each selected compound in each age group (%)					
Age group (years)					
1-5	169/1323 (13)	169/1111 (15)	167/1205 (14)	161/957 (17)	169/1597 (11)
5-20	171/1548 (11)	170/1958 (9)	170/1343 (13)	168/1946 (9)	168/1590 (11)
>20	167/1632 (10)	168/1218 (14)	168/1458(12)	167/1232 (14)	170/1990 (9)

In the table above average family size, range of family size and percentage female in each were calculated on the basis of selected number of samples from each camp.

4.3.1. *Plasmodium vivax*

4.3.1.1. *P. vivax* infection prevalence

4.3.1.1.1. *P. vivax* prevalence by RDT

P. vivax prevalence measured by RDT was 4% (96/2522) when data from all camps was combined. The camp specific prevalence of *P. vivax* infection ranged from 0.4-9%, with highest prevalence of 9% in camp 3 and lowest of 0.4 in camp1 (Table 4.2).

4.3.1.1.2. *P. vivax* prevalence by PCR

As mentioned earlier only a sub-sample of the total set of samples were assayed to identify low-density parasite infection. All RDT positive samples and a random selection of 120 RDT negative samples (done by randomly picking each sample from a bag containing well-shuffled samples of a whole camp) from each camp were assayed making a total to 1051 samples altogether. Prevalence of *P. vivax* infection detected by PCR, estimated by bootstrapping was 12% with a range of 5-15%. Again as for RDTs, camp 3 had the highest prevalence of infection and camp 1 the lowest. *P. vivax* parasite carriage measured by PCR was approximately 3 times greater than measured by RDT (Table 4.2).

Table 4.2 prevalence of *P.vivax* infection and prevalence of antibody responses to combined antigens in each camp

<i>P.vivax</i>						
Camp	RDT		PCR(Bootstrap values)		(PvAMA+PvMSP)	
	+ve/number tested	%(CI)	+ve/number tested	%(CI)	+ve/number tested	%(CI)
1	2/507	0.4(0-1)	10/137	7 (4-12))	88/501	17.5 (14-21)
2	14/507	3(1-5)	19/137	10(4-14)	164/504	31.9 (28-36)
3	49/505	9(6-12)	79/505	15 (13-18)	240/505	45 (38-50)
4	10/496	2(0-4)	8/130	4(0-6)	143/495	26.8 (23-31)
5	21/507	3(1-4)	23/142	7(3-11)	97/507	17.2 (14-21)
Total	96/2522	4(3-4)	140/1051	12(10-15)	732/2,512	28 (27-30)

4.3.1.1.3. Prevalence of *P. vivax* infec on by age

Prevalence of parasite for each camp by age group is shown in figure 4.1. An increase in prevalence with age was not seen except in camp 2 when detected by RDT. In camp 3 and 5 the highest parasite prevalence is seen in middle age group (see appendix 2 for detail).

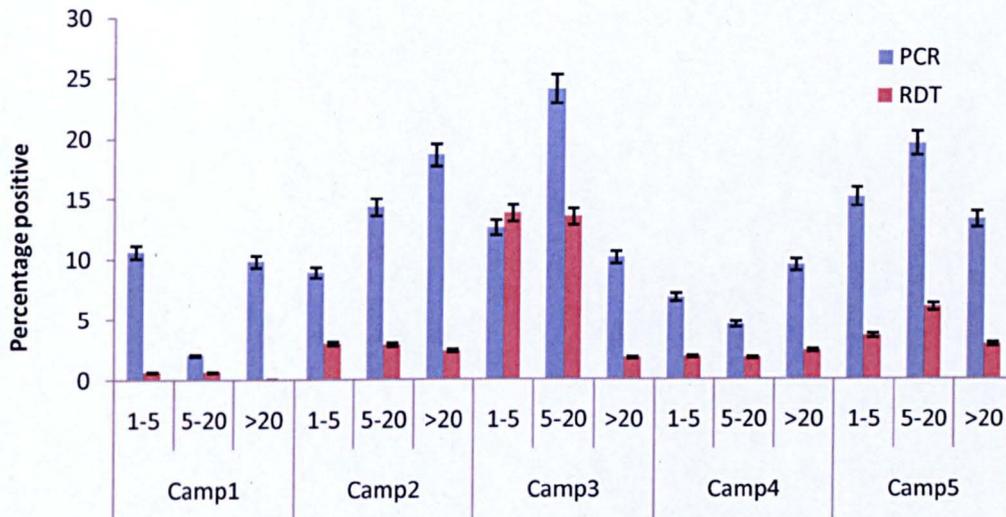


Figure 4.1 Prevalence of *P. vivax* infec on by age group in each camp

4.3.1.2. *Plasmodium vivax* serological responses

Of the 2522 samples collected ELISA was performed but 10 results were discarded for both antigens (AMA+MSP), an additional 11 were not included from AMA-1 and 10 from MSP-1₁₉. This resulted in a final of 2512 total for combined antigen, 2512 results for AMA-1 and 2518 data points for MSP-1₁₉. Missing ELISA results were discarded because of the discordant duplicate OD values (see chapter 3 for detail). They are unlikely to affect the results, as the discarded samples were few and did not appear to be systematically clustered i.e. come from one particular camp or house.

23% (590) individuals were seropositive to PvAMA-1 (see appendix 4), which was approximately double that of responses to PvMSP-1₁₉, 6 fold higher than parasite detected by RDT and approximately 1.7 times greater than PCR data. Seroprevalence was 11.5% (301) to PvMSP-1₁₉ (see appendix 4), which was 3 fold higher than RDT prevalence and similar to PCR parasite prevalence (12%).

To enhance the sensitivity of serological measure of transmission intensity antibody responses were combined for each species (AMA-1 and MSP-1₁₉), as previously done by Bousma *et al.* (Bousema *et al.*, 2010c). Antibody responses against *P. vivax* (AMA-1+MSP-1₁₉) were detected in 28% (732/2512) of individuals tested (Table 4.2) which is 7 fold higher than RDT and approximately double of the PCR prevalence.

Antibodies responses against *P. vivax* were significantly different among the camps ($\chi^2=27.2$, $p<0.01$). A consistency was observed among the three measures (serology, RDT and PCR), when heterogeneity in *P. vivax* infection and exposure among the camps was compared, which shows that for all three measures the highest malaria prevalence was observed in camp 3 and lowest in camp 1.

4.3.1.2.1. Age seroprevalence of *Plasmodium vivax*

Seroprevalence to *P. vivax* antigens by age group in each camp is summarized in Table 4.3. Seroprevalence to combined antigens (AMA+MSP) increased significantly with age, $p<0.01$ (using logistic regression). PvAMA-1 seroprevalence shows a clear increase with age in all studied sites with $p<0.01$, while the increase of seroprevalence for PvMSP-1₁₉ antibodies with age is not significant with $p<0.1$ (logistic regression) (for detail see appendix 4).

Table 4.3 Seroprevalence to combined *P. vivax* antigens in each age group in each camp

Camp	Age group	<i>P.vivax</i> (AMA+MSP)	
	(years)	+ve/ Number	% (CI)
Adezai (1)	1-5	6/169	3(0.5-5.4)
	5-20	25/170	13.5(7.5-19.5)
	>20	57/162	39.9(31.5-48.3)
Baghicha(2)	1-5	14/168	10.8(5-16.6)
	5-20	39/168	22.8(15.3-30.2)
	>20	111/168	64(56.6-71.6)
Jalala(3)	1-5	55/167	31.8(24-39)
	5-20	70/170	39.9(31.5-48.3)
	>20	115/168	66.8(59-74.6)
Kaghan(4)	1-5	15/161	9.1(4.4-14)
	5-20	29/167	15.6(9.5-21.8)
	>20	99/167	59.5(51.5-67.3)
Zangal patai(5)	1-5	23/169	11.6(6.5-16.7)
	5-20	32/168	17.6(11.2-24)
	>20	42/170	23.9(16.9-30.8)
Total	1-5	113/834	13.4(10.8-15.8)
	5-20	195/843	22.6(19.4-25.7)
	>20	424/835	52.9(49.4-56.4)

4.3.1.2.2. Age sero-prevalence curves of *Plasmodium vivax*

A model was fitted to the seroprevalence data using a reversible catalytic equation. The best fit was obtained using a common value of seroreversion rate called Rho (P), means one Rho was used which was not allowed to vary for each camp (single constraint Rho for all camps). A previous study in Tanzania also used one constraint Rho for all study sites to measure transmission intensity of malaria by using seroprevalence data and its comparison with EIR. It was assumed that a single constrained value of Rho could give a quality of fit of the model to the observed data and that individual values of Rho estimated for each location were also not significantly different, which was suggested that Rho is mostly independent of transmission intensity (Corran et al., 2007).

However, another study "The use of serological markers for evaluation of malaria and transmission dynamics" did not constrain Rho but allowed it to vary for each sentinel site (Cook, 2010). Therefore in this study seroprevalence curves were fitted with maximum likelihood model and 95% confidence intervals around the model across the five camps (Figure 4.2). Age seroprevalence curves for individual *P. vivax* antigen PvAMA and PvMSP-1₁₉ are given in appendix 7 and 8 respectively.

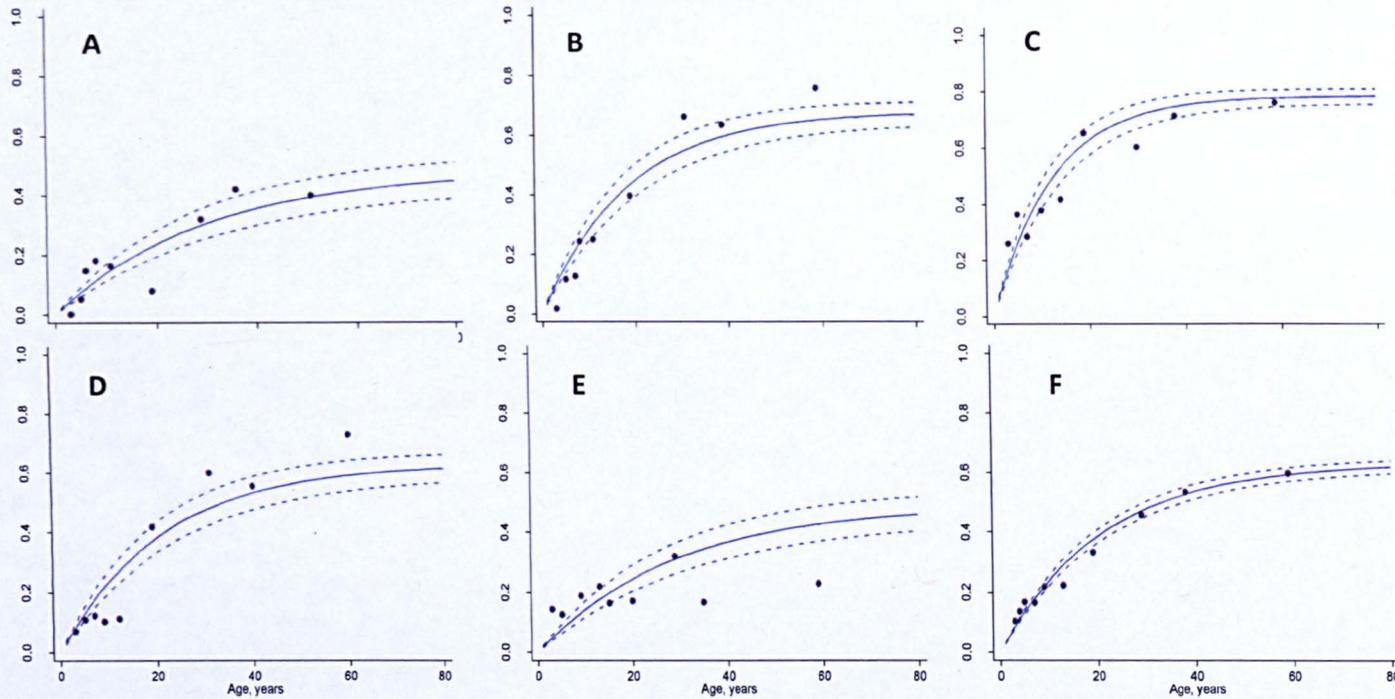


Figure 4.2. *P. vivax* age specific seroprevalence plots for *P. vivax* antibodies for each refugee camp

Graphs represent seroprevalence curves for A) camp1 B) camp2 C) camp3 D) camp4 E) camp5 and F) combined data of all five camps, which show the overall age seroprevalence pattern and transmission intensity of the study area. Filled circles represent observed data and the line is the fitted equation 1 in chapter 3 section 3.5.1.3. The proportion of seropositive individuals is represented by the vertical axis in each age group; the horizontal axis is the midpoint age of each age group, while dashed lines indicating 95% confidence intervals by likelihood ratio test.

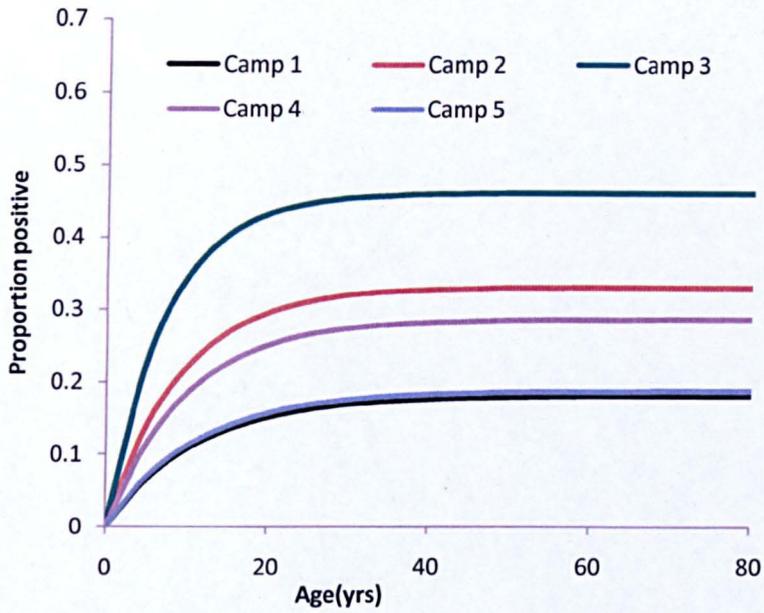


Figure 4.3 Comparison between age seroprevalence curves for an *P. vivax* antibodies among camps

The figure above is showing the age seroprevalence curves against combined *P. vivax* genes (AMA+MSP) of each camp to compare the heterogeneity in seroprevalence among camps.

4.3.1.2.3. SCR Vs. EIR

EIR was estimated previously by using SCR (λ) which was derived from age seroprevalence data (using single *P. falciparum* antigen AMA-1 or MSP-1₁₉) obtained from different MTI sites in Tanzania (Drakeley *et al.*, 2005). Following are the estimated EIR values based on the correspondence SCR values derived from seroprevalence to *P. falciparum* antigens from the above mentioned study.

Estimated EIR values based on SCR values calculated by using age seroprevalence to PfAMA-1

SCR (λ)	0.001	0.005	0.01	0.05	0.1	0.15	0.2
Estimated EIR	0.002	0.035	0.13	3	11	24	41

Same approach was used here to estimate EIR values for *P. vivax* for each camp. The resulted EIR values for the camps may not be as accurate as was calculated for Tanzanian data but may be indicative of EIR in the study sites. Firstly due to unavailability of the model based on *P. vivax* SCR values to estimate EIR. Secondly as mentioned above the estimation of EIR was made on the basis of using single *P. falciparum* antigen PfMSP-1₁₉ and I estimated SCR by combining antibody responses to both *P. vivax* antigens (estimated SCR values for individual *P. vivax* antigen are given in appendix 10).

Table 4.4 Seroconversion rates calculated for *P. vivax* using combined an gens (PvAMA+PvMSP) for each camp

Camp	<i>P. vivax</i>	
	SCR(CIs)	Estimated EIR's based on PfMSP-1 ₁₉
1	0.016(0.013-0.020)	0.3
2	0.036(0.031-0.043)	1.3
3	0.063(0.054-0.073)	4.4
4	0.029(0.025-0.035)	0.9
5	0.017(0.014-0.021)	0.3
Overall	0.030(0.028-0.032)	0.9

According to the table the es mated EIR (based on PfAMA-1 SCR) in the study area ranged between 0.3-4.4 by using seroprevalence data to combined *P. vivax*an gens (AMA-1+MSP-1₁₉).

4.3.1.2.4. *Plasmodium vivax* antibody titres by age groups and camps

Figure 4.4 shows median titre responses to PvAMA-1 and PvMSP-1₁₉ by age groups in each camp. There is a very clear increase of median titre responses with the age group using oneway ANOVA test. The results show, camp 1 with $F= 61.41$, $p<0.01$, camp 2 with $F= 138.8$, $p<0.01$, camp 3 with $F= 53.84$, $p<0.01$, camp 4 with $F= 108.6$, $p<0.01$ and camp 5 with $F=31.81$, $p< 0.01$) in case of PvAMA-1 (Figure 4.4. A). Again PvMSP-1₁₉ median titres show no increase with increasing age groups (camp1 with $F=3.08$, $p< 0.05$; camp 2 with $F=0.70$, $p<0.5$; camp 3 with $F=3.13$, $p<0.05$; camp 4 with $F=1.84$, $p<0.2$ and camp 5 with $F=0.89$, $p<0.5$) (figure 4.4.B).

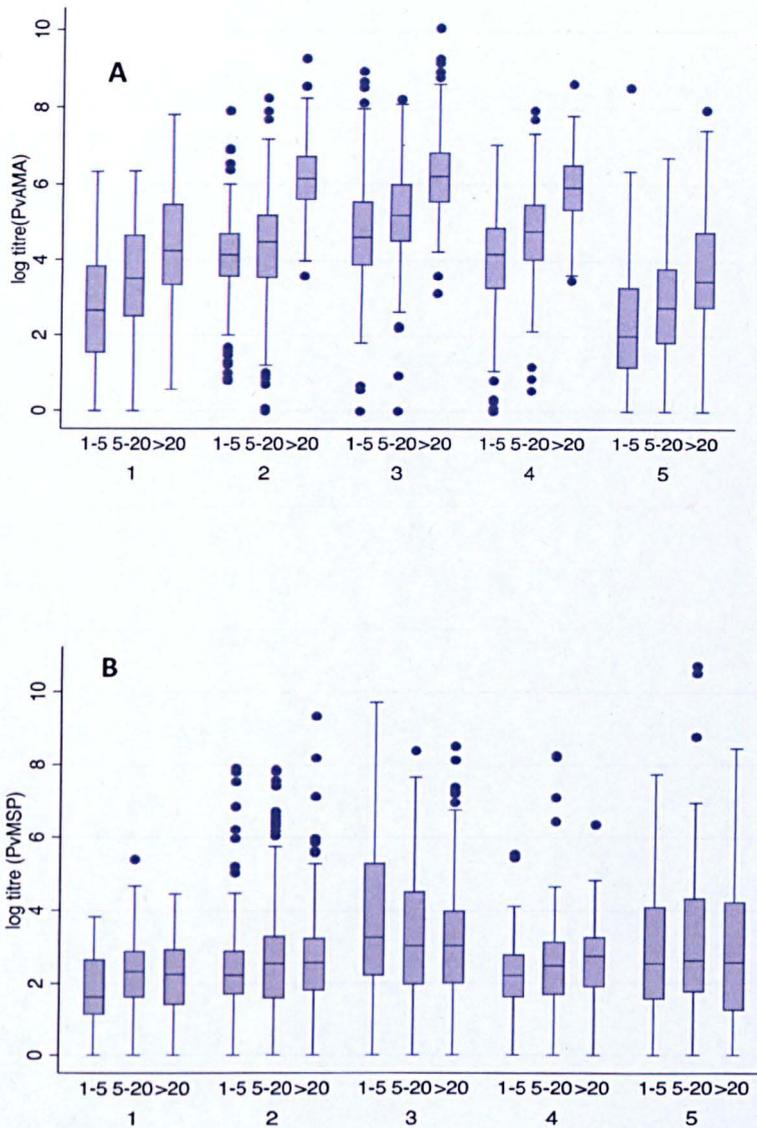


Figure 4.4 MedianPvAMA-1(A) and PvMSP₁₁₉ (B) titres by age group over camps

Box plot is showing the relationship between antibody titres against (A) PvAMA-1 (B) PvMSP-1₁₉ and age. The median titre is shown as middle horizontal line in the box. The 75th and 25th percentiles are represented by the top of box above the median and the bottom of the box below the median line respectively. Maximum and minimum titre values are shown by the whiskers of the plot. The PvAMA-1 titre shows a clear increase with increasing age, while PvMSP-1₁₉ also shows increase of titres with age except from camp3. Antibody titres are log transformed.

4.3.2. *Plasmodium falciparum*

4.3.2.1. *Plasmodium falciparum* prevalence by RDT

Only one study participant in camp 5 was *P. falciparum* positive making 0.2% (1/507) prevalence, while in the rest of the four camps the prevalence was zero. The overall *P. falciparum* infection prevalence detected was 0.04% (1/2522) (Table 4.5).

4.3.2.2. *Plasmodium falciparum* prevalence by PCR

The same samples (n=1051) assayed for *P. vivax* infection by PCR were also tested for *P. falciparum* (Snounou et al., 1993a). Prevalence of *P. falciparum* was 0% in three of the camps with one positive individual 0.8% (1/130) in camp 4 and eight infection positive individuals 1.4% (8/505) in camp 3. Overall prevalence detected by PCR was approx. 0.8% (9/1051) when all camps data was combined (Table 4.5).

4.3.2.3. *Plasmodium falciparum* serological responses

Of the 2522 samples assayed, 2514 samples were included in the analysis and the rest were discarded due to discordant ODs as described previously in chapter 3.

Overall seroprevalence to PfAMA-1 observed was 4.34 % (105), while to PfMSP-1₁₉ was 2%(48) individuals were seropositive. The seroprevalence data suggests that the residents of camp 2 (9.06%) have higher exposure to PfAMA-1 and camp 4 (1.44%) have lowest among the camps, while in case of PfMSP-1₁₉ the highest prevalence was observed in camp 5 and the lowest was in camp 3 (see detail of individual *P. falciparum* antigens in appendix 3). The data suggests low overall responses to both *P. falciparum* antigens, with antibody responses to PfAMA-1 (4.3%) were approx. 2 times higher than PfMSP-1₁₉ (2.0%), consistent with previous observations (Cook, 2010).

Table 4.5 shows seroprevalence data of *Plasmodium falciparum* to combined PfAMA-1 and PfMSP-1₁₉ antigens, where overall 6%(147) individuals were seropositive. By comparing seroprevalence among the camps, camp 5 residents show the highest (11%) antibody prevalence and camp 4 the lowest (3%) seroprevalence to combined PfAMA-1 and PfMSP-1₁₉. Parasite prevalence detected by PCR was 6 fold lower than the combined *P. falciparum* antigen seroprevalence, which ranges from 0-1.39%, with highest prevalence in camp 3 (Table 4.5).

Table 4.5 Percentages of study popula on seroposi ve to *P.falciparum* and prevalence infec on measured by RDT and PCR

<i>P. falciparum</i>						
Camp	RDT		PCR		(AMA+MSP)	
	Number tested	%(+ve)	Number tested	%(+ve)	+ve/Number tested	% (CI)
1	507	0	137	0	16/501	4 (2-6)
2	507	0	137	0	46/504	9 (7-11)
3	505	0	505	1.39(7)	23/505	4 (2-5)
4	496	0	130	0.77(1)	12/496	3 (1-4)
5	507	0.2(1)	142	0	50/507	11 (7-15)
Total	2522	0.04(1)	1051	0.76(8)	147/2,513	6 (5-7)

4.3.2.3.1. Age seroprevalence of *P. falciparum*

Overall, seroprevalence of antibodies to the two *P. falciparum* antigens was very low. The data do not show a clear increase with sero-positivity and age in any camp except camp 2 (Table 4.6) where seroprevalence significantly increases with increasing age $p > 0.001$ (logistic regression). Estimated SCR values of each *P. falciparum* antigen are given in appendix 9.

Table 4.6 Seroprevalence to combined *P. falciparum* antigens in each age group in each camp

Camp	Age group (years)	<i>P.falciparum</i> (AMA+MSP)	
		+ve/ Number	Percent (CI)
Adezai(1)	1-5	4/167	2.3(0-5.1)
	5-20	5/168	4.0(0-8)
	>20	7/166	5.5(1-9.3)
Baghicha(2)	1-5	2/168	1.7(0.0-4.0)
	5-20	9/168	5.2(1.0-9.4)
	>20	35/168	20.7(14.4-27)
Jalala(3)	1-5	6/167	2.6(0.4-4.7)
	5-20	9/170	3.9(1-6.5)
	>20	8/168	4.3(1.3-7.5)
Kaghan(4)	1-5	1/161	0.5(0-1.4)
	5-20	5/168	3.4(0.0-7.0)
	>20	6/167	4.6(0.8-8.0)
Zangal Patai(5)	1-5	17/169	11.0(5.4-16.0)
	5-20	17/168	13.0(6.4-19.0)
	>20	16/170	8.5(4.0-12.0)
Total	1-5	30/832	3.1(1.9-4.3)
	5-20	45/842	5.9(3.9-7.8)
	>20	72/839	8.9(6.9-10.9)

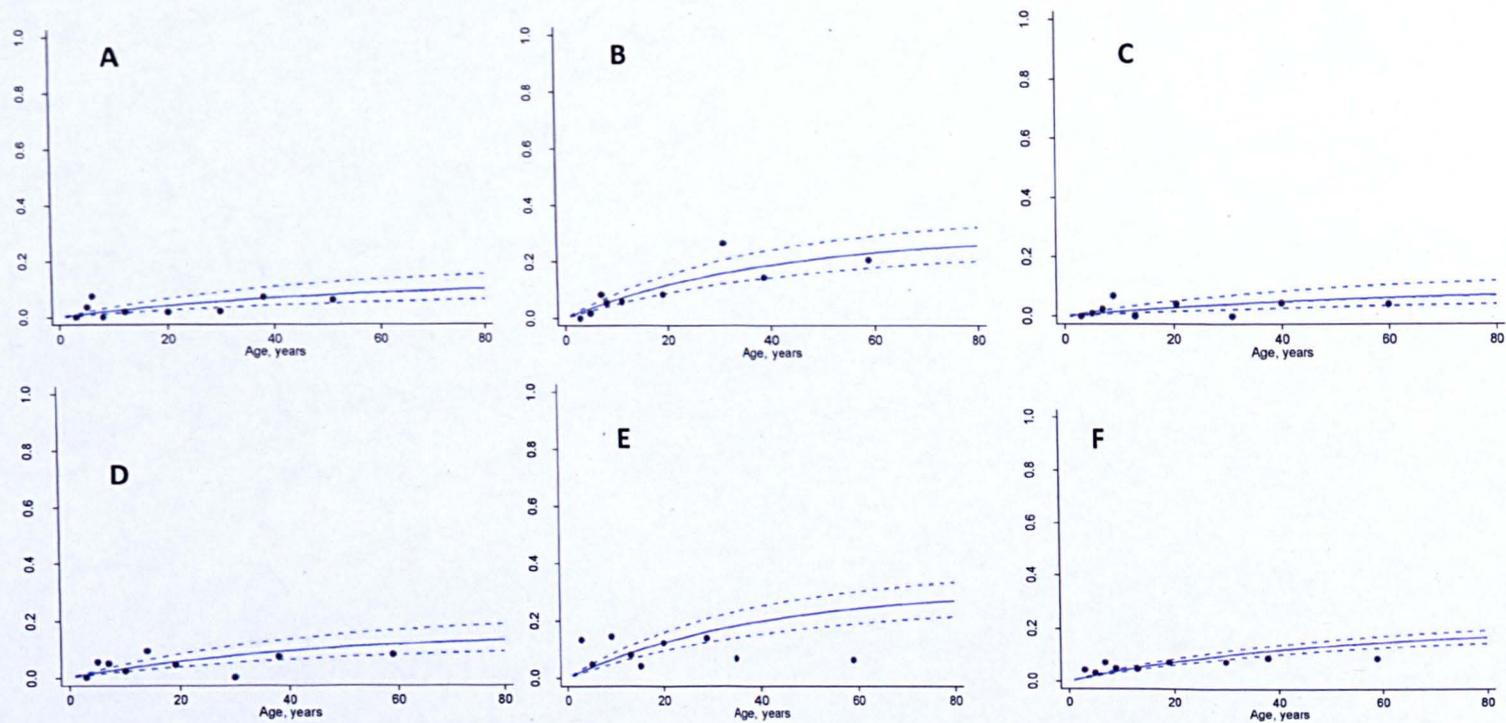


Figure 4. *SP.falciparum* age specific seroprevalence plots for each refugee camp

Graphs represent seroprevalence curves for A) camp1 B) camp2 C) camp3 D) camp4 E) camp5 and F) combined data of all five camps, which show the overall age seroprevalence pattern and transmission intensity of the study area. Filled circles represent observed data and the line is the fitted equation 1 in chapter 3 section 3.5.1.3. The proportion of seropositive individuals is represented by the vertical axis in each age group; the horizontal axis is the midpoint age of each age group, while dashed lines indicate 95% confidence intervals by likelihood ratio test.

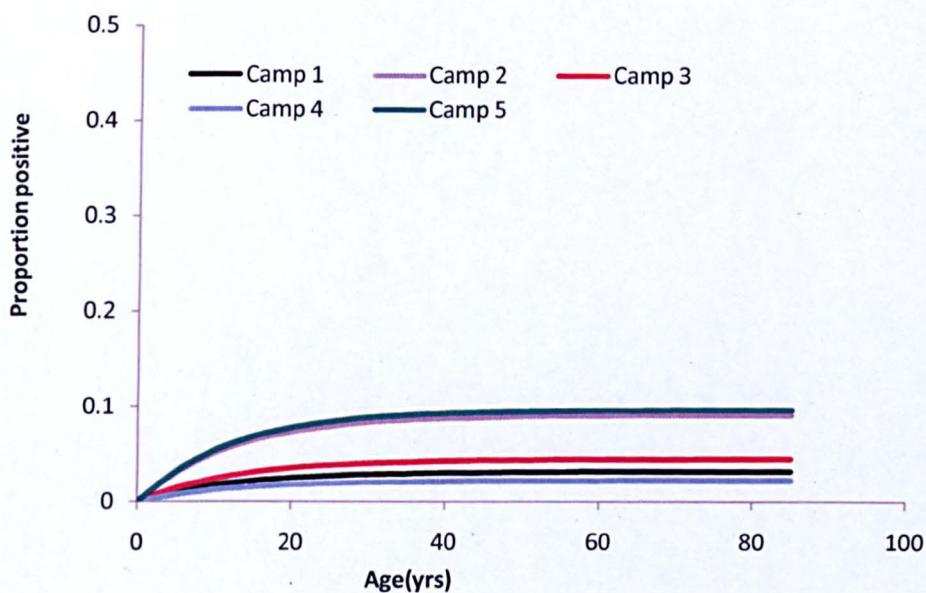


Figure 4.6 Comparison between aged age seroprevalence curves for an *P. falciparum* antibodies among camps

The figure shows the aged age seroprevalence curves against combined *P. falciparum* genes (PfAMA+PfMSP) of each camp to compare the heterogeneity in seroprevalence among camps.

Table 4.7 Estimated seroconversion rates (SCR) for *P. falciparum* using combined genes (PfAMA+PfMSP) for each camp

Camp	<i>P. falciparum</i>	
	SCR(CIs)	Estimated EIR's based on PfMSP-1 ₁₉
1	0.002(0.002-0.004)	0.003
2	0.007(0.005-0.009)	0.040
3	0.003(0.002-0.005)	0.007
4	0.002(0.001-0.003)	0.003
5	0.008(0.006-0.011)	0.060
Overall	0.005(0.004-0.005)	0.030

4.3.2.3.2. *P. falciparum* body titres and age groups by camp

Figure 4.7.A suggest no clear pattern of increase in median values of titres for PfAMA-1 with increasing age, but except for camp1 the median titre shows little increase with age. According to Figure 4.7.B, a very little increase in median the PfMSP-1₁₉ antibodies titre values with age group in case of camp 1 but again no clear increase of titres with age in case of other four camps (2-5) was observed. The detail of oneway ANOVA is given below:

PfAMA-1: Camp 1 with $F= 16.3$, $p<0.01$; camp2 with $F= 13.53$, $p<0.24$; camp 3 with $F= 0.36$ $p<0.7$; camp 4 with $F= 6.18$, $p<0.02$ and camp 5 with $F= 0.73$, $p<0.5$ (figure 4.4. B).

PfMSP-1₁₉: Camp 1 with $F= 12.53$, $p<0.01$; camp 2 with $F= 1.51$, $p<0.3$; camp 3 with $F= 4.46$, $p<0.01$; camp 4 with $F=3.08$, $p<0.05$ and camp5 with $F= 0.107$, $p=0.9$ (figure 4.4. B).

Camp 5 median antibody titre was very low (about two third of the individuals had negative antibody titre) which is not consistent with median antibody titre with other four camps. To check the validity of the camp 5 data the ELISA assay and analysis were repeated twice but similar results were obtained. These unusual results for camp 5 may be due to handling of the blood spots during collection and storage or may be due to other reasons.

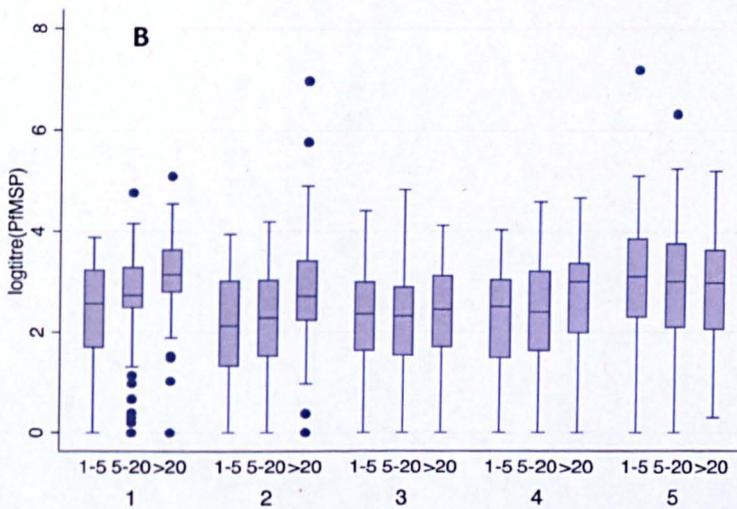
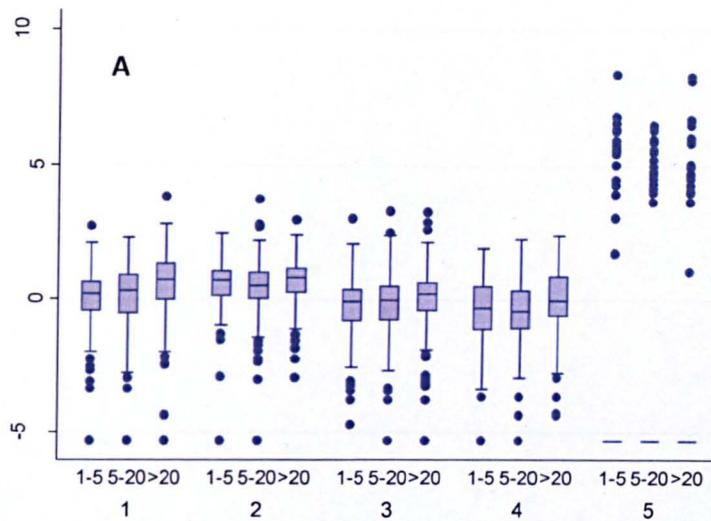


Figure 4.7 Median titres of antibodies to (A) PfAMA-1 and (B) PfMSP-1₁₉ in different age groups in each camp

4.3.3. Comparison of serological and parasitological measures

4.3.3.1. *Plasmodium vivax*

RDT vs PCR

A comparison of the performance of PCR and RDT in detecting *P. vivax* infection is shown in Table 4.8. As expected the nested PCR test identified more positive infections than by RDT and it was used as the gold standard to calculate the sensitivity of RDT. The RDT had shown a sensitivity of 81.1% (95% CI: 71.7%-88.4%) and a specificity of 93.4% (95% CI: 91.6%-94.9%). A positive predictive value (PPV) of 55% (95% CI: 46.4%-63.4%) and a negative predictive value (NPV) of 98% (95% CI: 96.9%-98.8%) with observed ROC value 0.9.

Table 4.8 Comparison of the performance of RDT using PCR as a standard

<i>P. vivax</i>	RDT		Total
	Pos.	Neg.	
Pos.	77	63	140
Neg.	18	893	911
Total	95	956	1,051

<i>P. vivax</i>	Sensi vity (95% CI)	Speci city (95% CI)	PPV (95% CI)	NPV (95% CI)	ROC (95% CI)
RDT	81.1 (71.7-88.4)	93.4 (91.6-94.9)	55 (46.4-63.4)	98 (96.9-98.8)	0.9 (0.8-0.9)

- Sensi vity is the propor on of diseased pa ents correctly iden ed. Speci city is the propor on of healthy pa ents correctly iden ed.
- ROC (Receiver Opera ng Characteris c curve) area is the average of sensi vity and speci city
- PPV & NPV (posi ve and nega ve predic ve values) show the probability of pa ent having the disease with a posi ve test or not having the disease with a nega ve test respec viely.

4.3.3.1.1. RDT vs. An bodies responses

P. vivax prevalence measured by RDT was compared with the immune response against *P. vivax* antigens (Table 4.9). 59.4% of RDT positive individuals had antibodies to one or other *P. vivax* antigens (AMA+MSP). 35.4% of the PvAMA-1 seropositive samples were RDT positive, while 53.9% individuals of RDT positive were PvMSP-1₁₉ positive (see appendix 12). These data suggest that the sensitivity (serological detection of parasite positive samples) of PvMSP-1₁₉ was higher than PvAMA-1. 23%, 11% and 28% of the RDT negative individuals were seropositive respectively to AMA-1, MSP-119 and combined (AMA+MSP).

4.3.3.1.2. PCR VS. An body responses

By comparing *P. vivax* immune responses with parasite prevalence measured by PCR, 32.2 % parasite positive individuals were detected by PvAMA-1. 39.9 % of PvMSP-1₁₉ seropositive individuals were parasite positive. It was evident from the data that PvMSP-1₁₉ is more sensitive to detect parasite positives individuals than PvAMA-1 (detailed in appendix 13). In case of PvMSP-1₁₉ significantly ($p < 0.001$) more parasite positive individuals were seropositive than seronegative, while for PvAMA-1 more parasite positive by both PCR and RDT individuals were seronegative.

By combining both antigens, the sensitivity of the ELISA assay was increased from 32.2%(PvAMA-1) and 39.9% (PvMSP-1₁₉) to 50.0% to detect parasite positive individuals (PCR), which were more likely seropositive with $P < 0.01$ (chi squared test). 50% of the parasite positive individuals detected by PCR were seropositive against both *P. vivax* antigens (AMA+MSP) and 33.9% individuals seropositive to both antigens were parasite negative (Table 4.10).

Table 4.9 Number and percentages of RDT positive and anti-*P. vivax* seropositive individuals

Combined (AMA+MSP)	RDT negative	RDT positive	P value
Seronegative	72.06 %(1741)	40.62 %(39)	<0.01
Seropositive	27.94 %(675)	59.38 %(57)	

Table 4.10 Number and percentages of PCR positive and seropositive individuals against combined *P. vivax* antigens

Combined (AMA+MSP)	PCR negative	PCR positive	P value
Seronegative	66.01%(600)	50%(70)	<0.01
Seropositive	33.99 %(309)	50%(70)	

4.3.3.1.3. Association of *P. vivax* prevalence measured by RDT and PCR and *P. vivax* seroconversion rate (SCR) in camps

The data suggest a strong association between SCR (estimated from PvAMA-1 seroprevalence data) and RDT ($R^2=0.6$) but the association was found to be very weak for PCR ($R^2=0.2$). While PvMSP-1₁₉ prevalence has a strong association with RDT ($R^2=0.9$) and moderate with PCR ($R^2=0.5$). The correlation between SCR (estimated from combined antibody responses to both antigens, i.e. (PvAMA+PvMSP) and parasite prevalence (RDT and PCR) was also moderate ($R^2=0.5$). The scatter plots of relationship between infection prevalence and SCR are given in Figure 4.10 and R squared values are given in table below.

R ² value		
<i>P. vivax</i>	RDT	PCR
AMA	0.6	0.2
MSP	0.9	0.5
AMA+MSP	0.5	0.5

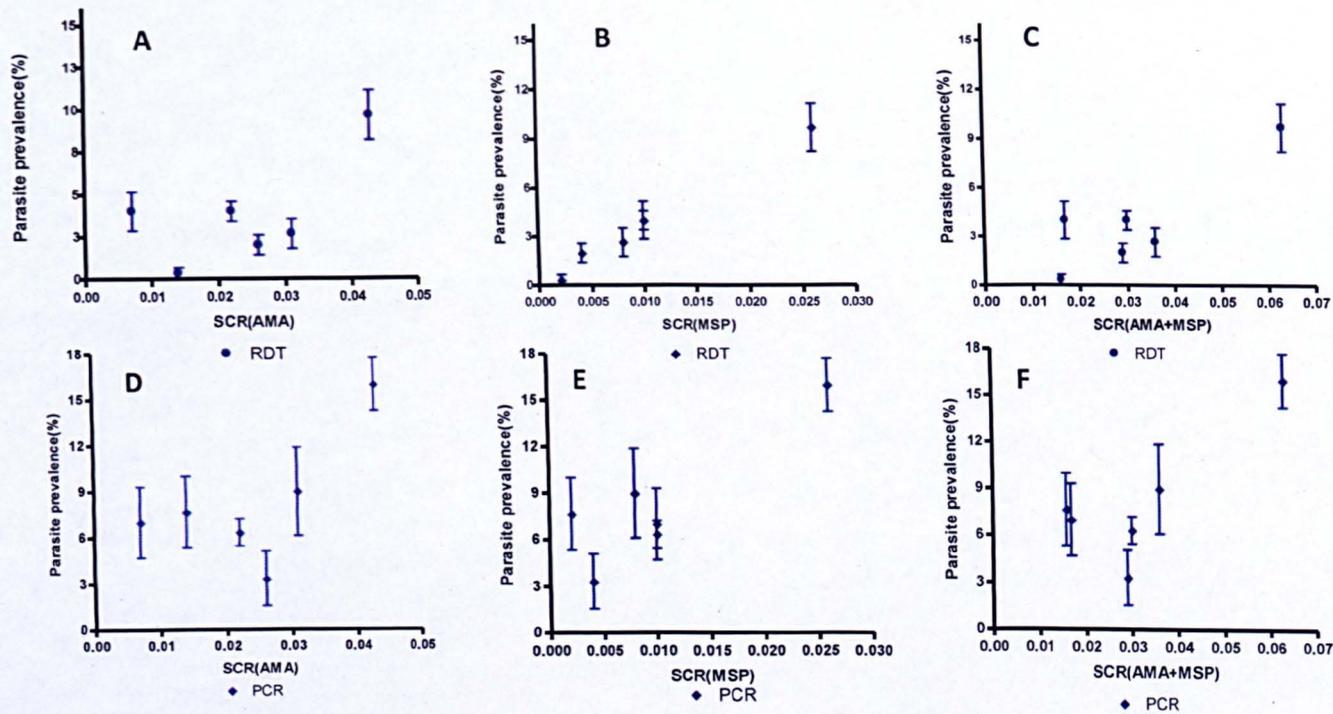


Figure 4.8 Relationship between *P. vivax* infection prevalence (measured by RDT and PCR) and seroconversion rate (SCR) in camps

The above scatter plots show the association between prevalence of *P. vivax* infection measured by RDT and PCR and SCR estimated from age seroprevalence of PvMSP-1₁₉ and PvAMA-1. Plot A to C represents relationship between *P. vivax* infection measured by RDT and SCR of PvAMA-1 (A), PvMSP-1₁₉ (B), PvAMA-1+PvMSP-1₁₉ (C) and plots D to F represents association between *P. vivax* infection measured by PCR and SCR of PvAMA-1 (D), PvMSP-1₁₉ (E), PvAMA-1+PvMSP-1₁₉ (F).

4.3.3.1.4. Prevalence of infec on VS. Es mated EIR

The scatter plots (figure 4.9) show the association between estimated EIR based on SCR values and prevalence of *P. vivax* infection measured by RDT and PCR, the relationship was linear ($R^2=0.7$) and ($R^2=0.6$) respectively.

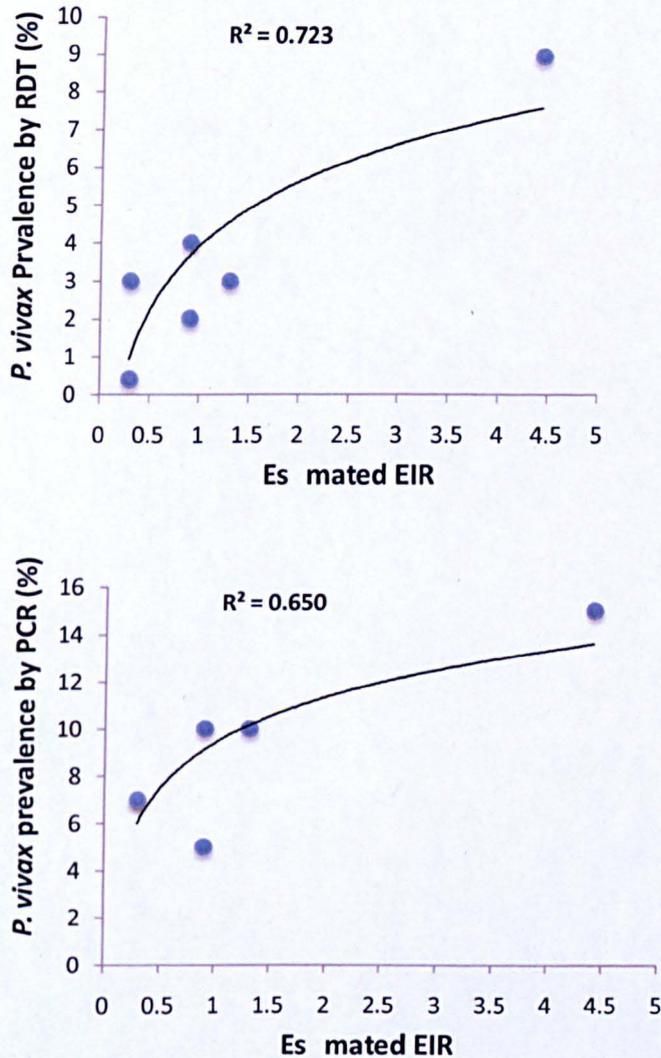


Figure 4.9 Relationship between es mated entomologic inocula on rates (EIRs) based on the SCR plotted on linear scale and prevalence of infection of *Plasmodium vivax* measured by both RDT and PCR.

4.3.3.1.5. Comparison between parasite prevalence and antibody titres against *P. vivax* in each age group

The data shows higher PvAMA-1 and PvMSP-1₁₉ titres in individuals who were RDT and PCR positive compared to negative samples (figure 4.10 A and B).

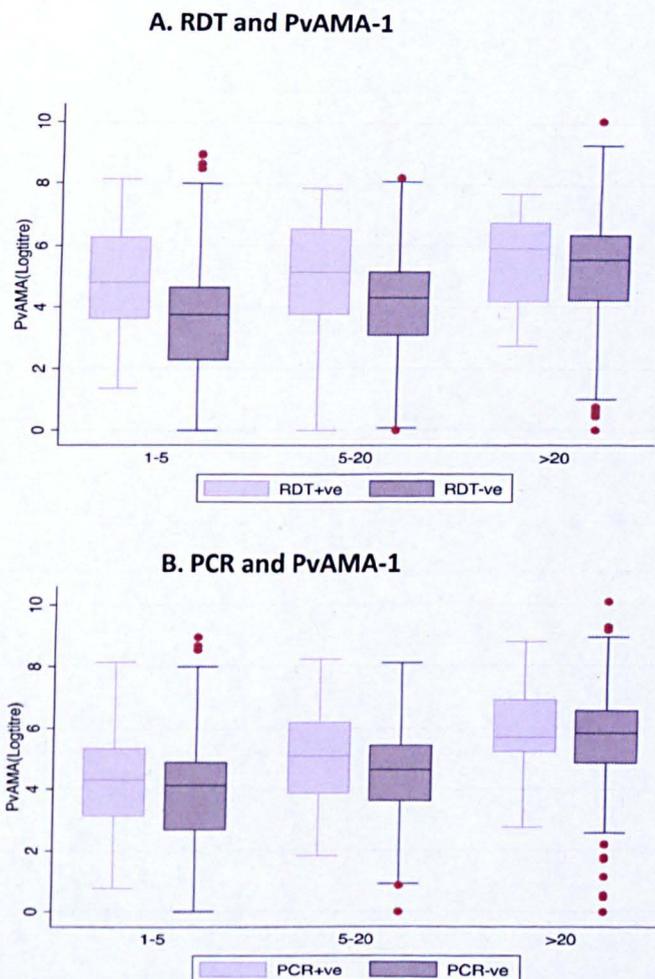


Figure 4.10 Box plot showing relationship between antibody titres PvAMA-1 and (A) RDT, (B) PCR prevalence by age group

The middle horizontal line of the bar represents the median of the log transformed antibody titres, while the top of the box above the median is 75th percentile and below the median the bottom of the box is 25th percentile. Both boxes represent 50% of the antibody titres. The maximum and minimum antibody titres are the whiskers of the plot. Antibody titres are log transformed.

Again antibody response (log transformed titres) to PvMSP-1₁₉ in RDT/PCR positive is higher than RDT/PCR negative individuals.

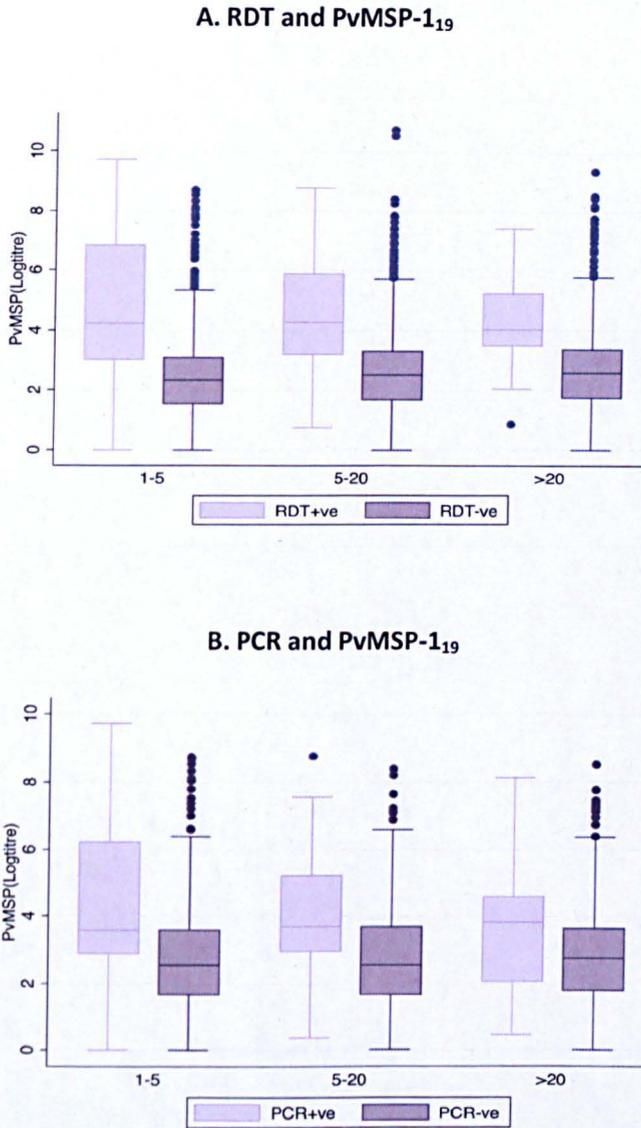
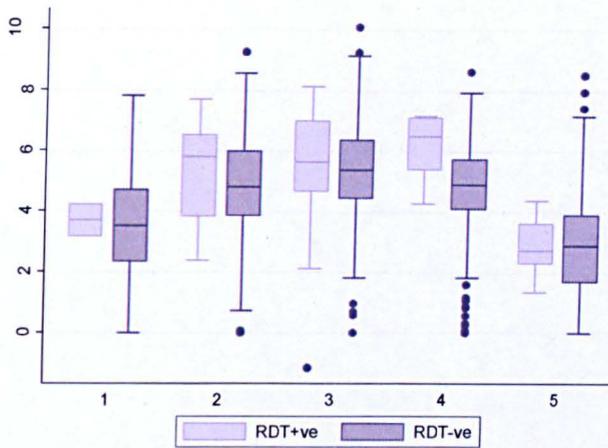


Figure 4.11 Box plot showing relationship between antibody titres of PvMSP and (A) RDT and (B) PCR prevalence by age group

The middle horizontal line of the bar represents the median of the log transformed antibody response, while the top of the box above the median is 75th percentile and below the median the bottom of the box is 25th percentile. Both boxes represent 50% of the antibody titres. The maximum and minimum antibody titres are the whiskers of the plot. Antibody titres are log transformed.

A. RDT and PvAMA-1



B. RDT and PvMSP-1₁₉

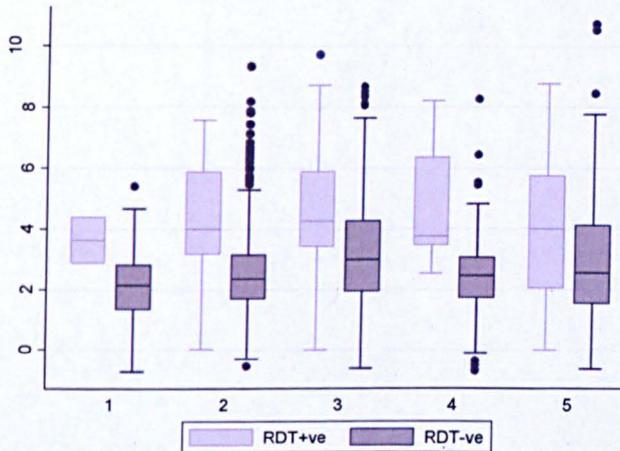
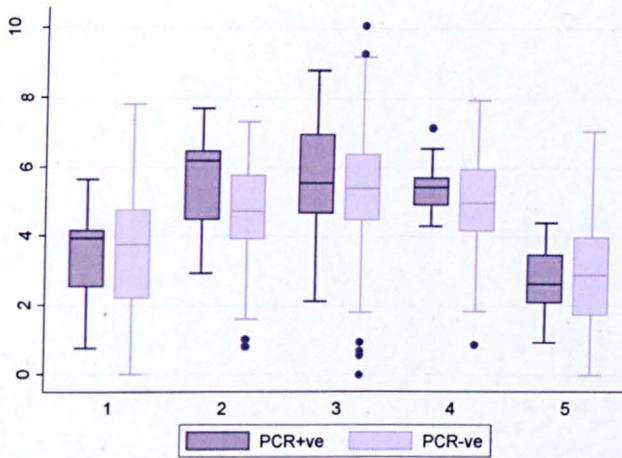


Figure 4.12 Comparison between parasite prevalence measured by RDT and an body tres against (A) PvAMA-1 and (B) PvMSP-1₁₉ bycamp

The middle horizontal line of the bar represents the median of the log transformed an body tres, while the top of the box above the median is 75th percent le and below the median the bo om of the box is 25th percent le. Both boxes represent 50% of the an body tres. The maximum and minimum an body tres are the whiskers of the plot. An body tres are log transformed.

A. PCR and PvAMA-1



B. PCR and PvMSP-1₁₉

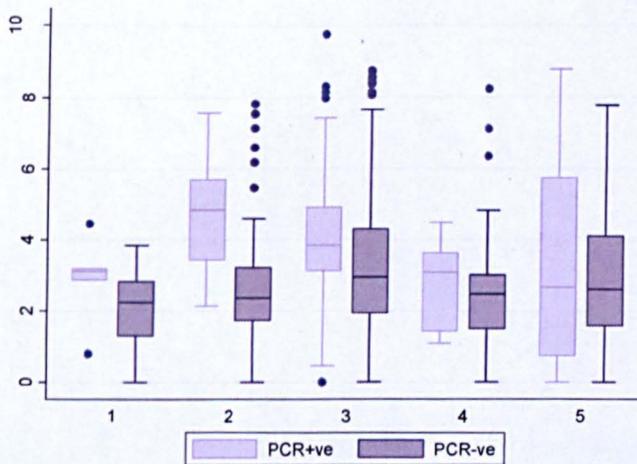


Figure 4.13 Comparison between parasite prevalence (measured by RDT/PCR) and an body tres (PvAMA and PvMSP) bycamp

The middle horizontal line of the bar represents the median of the log transformed an body res, while the top of the box above the median is 75th percent le and below the median the bo om of the box is 25th percent le. Both boxes represent 50% of the an body tres. The maximum and minimum an body tres are the whiskers of the plot. Where an body tres are log transformed.

4.3.3.2. Association between serological and parasitological measures for *P.falciparum* in Afghan refugee camps of KP

Due to low prevalence of *P. falciparum* clear relationships between parasite prevalence and seroprevalence were difficult to observe. The figures show relationship of parasite prevalence (measured by RDT and PCR) in all age groups and SCR are given in appendix 11.

4.3.4. Seroprevalence patterns between *P.falciparum* and *P.vivax*

67.9% of the studied population was seronegative to all antigens used and 2.9% were seropositive to both *P. falciparum* and *P. vivax* antigens (Table 4.11). In case of PfMSP-1₁₉ and PvMSP-1₁₉ 0.3% individuals were seropositive to both antigens, while 2.2% individuals were positive to both AMA-1 antigens (PfAMA-1 and PvAMA-1). 0.2% of studied people were seropositive to both antigens of falciparum (Table 4.13), while 6.3% individuals were seropositive to both vivax antigens (Table 4.12).

Table 4.11 Percentages of the studied population seropositive to neither, either or both species

N=2,508	<i>P. vivax</i> (AMA+MSP)	
<i>P. falciparum</i> (AMA+MSP)	Seronegative	Seropositive
Seronegative	67.9(17020)	26.3(659)
Seropositive	3(74)	2.9(73)

Table 4.12 Percentages of the studied population seropositive to neither, either or both *P. vivax* antigens

N=2,513	PvMSP-1 ₁₉	
PvAMA-1	Seronegative	Seropositive
Seronegative	70.7(1777)	5.7(142)
Seropositive	17.2(432)	6.3(158)

Table 4.13 Percentages of the studied population on seropositive to neither, either or both *P. falciparum* genes

N= 2,514	PfMSP	
PfAMA	Seronegative	Seropositive
Seronegative	92.9(2335)	1.7(42)
Seropositive	3.8(95)	0.2(6)

In this study 6.45%(8) individuals *P. vivax* parasite positive detected by RDT (Table 4.14) and 9.5%(16) *P. vivax* PCR were seropositive to *P. falciparum* antigens (Table 4.15).

Table 4.14 Number and percentages of *P. vivax* RDT positive and seropositive individuals against, an -*P. falciparum*

<i>P. falciparum</i>	<i>P. vivax</i>		
(AMA+MSP)	RDT negative	RDT positive	P value
Seronegative	94.3 %(2278)	93.6 %(88)	0.7
Seropositive	5.7 %(139)	6.4 %(8)	

Table 4.15 Number and percentages of *P. vivax* PCR positive and seropositive individuals against, an -*P. falciparum*

<i>P. falciparum</i>	<i>P. vivax</i>		
(AMA+MSP)	PCR negative	PCR positive	P value
Seronegative	95.6%(866)	90.5%(124)	0.01
Seropositive	4.4 %(43)	9.5%(16)	

4.3.4.1. Cross reactivity between the equivalent antigens of *P. vivax* and *P. falciparum*

Scatter plots of PfAMA-1 vs. PvAMA-1 (Figure 4.14) and PfMSP-1₁₉ vs. PvMSP-1₁₉(Figure 4.15) were used to check the credibility and validity of the data, whether there is cross reactivity between the antigens. Antibody responses against equivalent antigens of *P. vivax* and *P. falciparum* obtained from the dataset of this study do not show any evidence of cross reactivity.

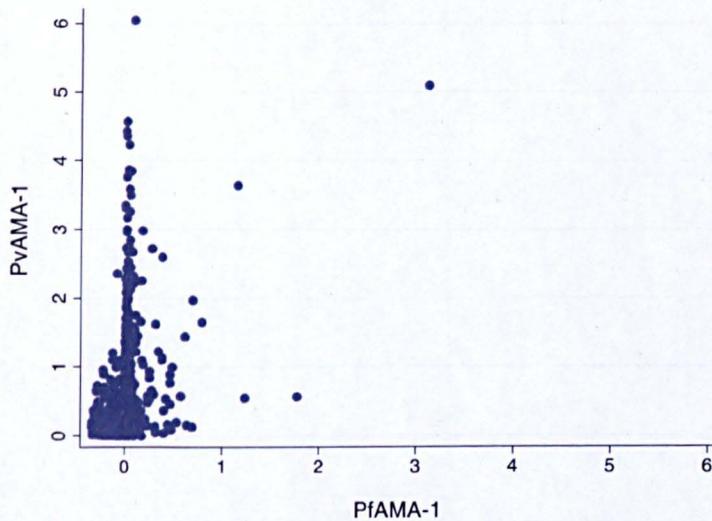


Figure 4.14 Scatter plot shows relationship between PvAMA-1 and PfAMA-1 (ODs)

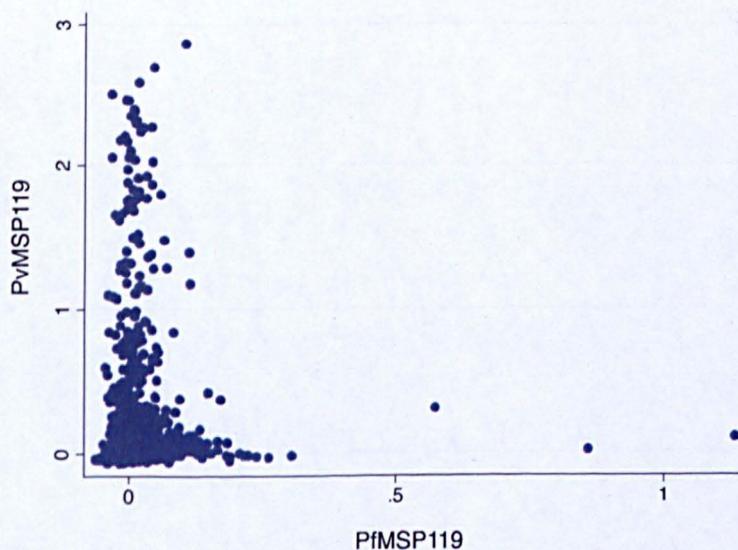


Figure 4.15 Scatter plot shows relationship between PvMSP-1₁₉ and PfMSP-1₁₉ (ODs)

4.4. Discussion

Results presented in this chapter are from the malaria epidemiological study undertaken in the Afghan refugee camps designed to compare different methods for estimating malaria transmission intensity in the different camps. The study included measures of parasite infection (RDT and PCR) and serological prevalence of both *P. falciparum* and *P. vivax* malaria in all age groups. The main reason of this study was to use serological and parasitological measures to provide up-to-date information on exposure to malaria and infection in the study area and compare camps on the basis of these measures. Transmission intensity observed in this area is very low making it potentially suitable for malaria elimination. However, there are little accurate, current data available and any new data will help guide and plan control efforts.

The field investigations of parasite prevalence included CareStart™ Malaria HRP-2/pLDH (Pf/pan) Combo Test detecting the *Plasmodium falciparum* specific antigen histidine-rich protein (HRP-2) and the pan-*Plasmodium* antigen lactate dehydrogenase (pLDH). All the RDT used in the field worked according to the information provided by the manufacturer except for only three RDT cassettes, which I repeated. A sub set of 120 RDT negative samples and all RDT positive were tested for parasite infection by PCR (Snounou et al., 1993a), to measure submicroscopic infection and to validate RDT results. All samples underwent serological analysis for antibody responses to AMA-1 and MSP-1₁₉ against both *P. falciparum* and *P. vivax*.

4.4.1. *Plasmodium vivax*

4.4.1.1. Prevalence of infection measured by RDT and PCR

The overall prevalence of *P. vivax* infection detected by RDT was 4% and ranged from 4.0-9.0%, being highest in camp 3 and lowest in camp1. This difference in the prevalence of *P. vivax* infection in different studied camps (when measured by RDT) is highly significant with $p < 0.0001$ (pearson's χ^2). While the overall prevalence of *P. vivax* infection measured by PCR observed was 12%, which ranged from 4% (camp 4) to 15% (camp3). The difference in the *P. vivax* prevalence among different camps is

weakly significant when measured by PCR ($p < 0.05$) and highly significant when estimated by RDT ($p < 0.01$).

One likely explanation for the observed differences in the prevalence of infection between the camps might be, the difference in the geographical location of the camps (close to rivers and rice fields etc.) and prevalence differences in vector abundance (Rowland and Nosten, 2001). Another possible explanation for the detected variation in the prevalence of vivax infection among the camps may be due to differences in the use of ITN, there prevalence and/or previous malaria control activities and malaria awareness in the camp. For example there were fewer people using ITN in the people of camp 3 and 5 as compared to other camps (this will be described in more detail in the following chapter).

Another likely reason for the observed heterogeneity in the prevalence of infection among camps may be the historically higher prevalence of malaria in some camps. This higher exposure may have led to development of partial immunity in the individuals, which is effective at controlling parasite densities. For example in the present study the camp (3) in which prevalence of infection was found to be high (9.0% by RDT), had lowest Annual Vivax Incidence (AVI) (BHU data) in the past as compared to other camps, while the camp (1) which has now lowest prevalence of infection (0.4% by RDT), was one of the camps experienced high Annual Vivax Incidence in past (BHU data appendix 14).

Although the surveys were carried out at different months of the year (June 23rd-September 19th, shown in table 1 of chapter 2) in different camps, but comparison of infection prevalence at different study sites was possible as the first cases of *P. vivax* malaria were observed to happen much earlier, mainly in April (Rowland et al., 1997a). However, the prevalence of infection observed for studied camps is consistent with the number of cases recorded at the BHU. These results also are in agreement with previous studies (Rowland et al., 2001), representing *P. vivax* the major species of malaria as compared to *P. falciparum* has been successfully reduced (Rowland et al., 2002b) after major epidemics seen in early 1990s in Afghan refugee camps.

PCR detected a higher number of parasite positive individuals than RDT as expected, which is consistent with other studies (Harris et al., 2010, Okell et al., 2012, Okell et al.,

2009). A review on the submicroscopic infections hypothesized that in lower transmission areas infections on average are older and more likely developed to submicroscopic phase, while in high-transmission areas repeated infections and superinfection may results in the increase of average parasite density in an infected individuals (Okell et al., 2012). Another likely explanation for the high prevalence of submicroscopic infections could be the frequency of G6PD deficiency in the Afghan population, which would reduce parasite growth. Finally high rates of infection may be due to location of the refugee camps along the rivers, on marshy places or rice fields that support vector breeding sites (Leslie et al., 2010).

In this study some of the RDT *P. vivax* positive individuals (18/94) were missed by PCR, similar findings are also reported by other studies (Osman et al., 2010). One of the likely reasons for the discordance between RDT and PCR might be the false positive results by RDT, as were reported in Sudan (Osman et al., 2010). According to some studies false positive RDT results (poor specificity) were due to cross-reactivity with rheumatoid factor in blood and heterophile antibodies, but replacement of IgG with IgM (malaria) in recent products are thought to reduce this problem (Wongsrichanalai et al., 2007). While some of the RDT are designed to detect antigen rather than live parasites, which means detecting previous exposure (older infection) rather than recent infection.

However, a study conducted in Afghanistan to evaluate the performance of three types of malaria RDT, showed 98.6% sensitivity and 0% false positive results (100% specificity) for the same type of RDT (CSPfPan) used in this study (Mikhail et al., 2011). Another study for the evaluation of malaria RDT in a reference setting recorded 90.2% sensitivity at a parasite densities 500/ μ l and 100% specificity for *P. vivax* (WHO, 2011b). According to these studies the malaria RDT used in the present study was sufficiently consistent and reliable, to make it appropriate for *P. vivax* predominant region.

Another alternative reason for the discordance between PCR and RDT might be the limited sensitivity of conventional PCR, which is affected by using small amount of blood used for detection and PCR sensitivity may also be affected by not removing PCR inhibitors (e.g. chelex) during DNA extraction. Some studies also reported DNA degradation due to enzymatic activities, which will affect PCR sensitivity (Rodulfo et al.,

2007). Stratifying by age group prevalence of infection (RDT and PCR) was higher in children (1-5) and in individuals with age 5-20, lowest in adults (20+years) as was expected (fig.4.1).

4.4.1.2. Serological Data

Since this study was to be conducted in a low transmission area the corresponding immune responses were expected to be low. Therefore to detect low level of exposure to parasite, the blood stage antigens AMA-1 and MSP-1₁₉ were selected based on previous studies (Drakeley et al., 2005, Corran et al., 2007, Bousema et al., 2010c, Cook, 2010, Cook et al., 2010). As discussed earlier in Chapter 3 blood stage antigens were found to be relatively more suitable for detection of low levels of exposure to malaria than pre-erythrocytic stage antigens (CSP and LSA) (Drakeley et al., 2005, Orlandi-Pradines et al., 2006, Bousema et al., 2010c, Cook, 2010).

To enhance the sensitivity of serological measure of MTI responses to both AMA-1 and MSP-1₁₉ (AMA+MSP) were combined as in a previous sero-epidemiological study conducted in Somaliland which is a similar low transmission area (Bousema et al., 2010c). We needed to measure antibody response to combined antigens so if an individual did not react to one antigen, then reaction to another antigen may be detected. This was done for both *P. vivax* and *P. falciparum* responses.

To minimize chances of cross reactivity between immune responses to malaria and other parasites, recombinant proteins (representing single antigens) were used as cross reactivity have been reported when whole parasite extract was used (Abramo et al., 1995, Naus et al., 2003). In this study no relation in antibody titers between the homologous antigens of *P. falciparum* and *P. vivax* was observed (Fig.4.14 and 4.15). Additionally tested samples at significantly higher dilutions (1/1000), as previous studies suggested less chances of cross reactivity at a high dilution (1:80) of sera (Bousema et al., 2010c, Abramo et al., 1995). Moreover, a suitable cutoff to differentiate seropositive individuals from seronegative, was derived from within the study sample to minimize bias caused by local cross-reactive antigens (Bousema et al., 2010c).

Defining cutoff values is critical for interpretation and the modeling approach to seroprevalence data used here. Ultimately the splitting of a population into antibody

positives and negatives is an arbitrary process, so it is important to make the criteria for this partition, as clear as possible, and to define when particular criteria will be appropriate. For example a cutoff value calculated using a mixture model needs a substantial proportion of a seronegative population in the antibody responses, which is less likely in high transmission areas as almost all individuals will have high antibody levels. In such a high transmission population the cutoff value will be overestimated, resulting in underestimation of seroprevalence. Some loss of sensitivity may also occur when including parasite positive individuals (some of whom may have developed low antibody responses in response to infection and in whom existing basal antibody levels will be enhanced). Since OD and titre distributions are usually quite asymmetrical, a slight change in cutoff in a population with few clearly positive individuals may result in a large change in the proportion of defined positives. The use of sample sets from malaria unexposed populations (often Caucasian) to establish parameters for normal unexposed distributions in populations from very different environments is also problematical as malaria exposed populations often differ markedly in other characteristics – total IgG levels in Africa, for instance, or exposure to a range of other parasites which may cross-react non-specifically with malaria antigens. Since groups of samples from malaria endemic populations which can be guaranteed to be malaria unexposed are difficult to identify let alone obtain, one or other of the two alternative approaches of modeling exposed populations or using controls of doubtful validity must be employed. Since exposure in Afghan refugee populations is relatively light as demonstrated by the parasite data, I have used mixture modeling, in which the unexposed population is assumed to have a normally distributed set of ODs (admittedly based on information from unexposed Caucasian populations) and the exposed population is approximated by a second Gaussian. Cutoffs are based solely on the parameters of the Gaussian fitted to the putative non-exposed individuals.

The cutoff estimation based on mixture model was suggested suitable in low transmission areas where majority of the population is supposed to be seronegative (Cook, 2010). Therefore this model was used to calculate cutoff in this study as the camps are coming under low transmission areas of Pakistan and existing BHU data suggests low incidence of clinical disease too.

4.4.1.2.1. Seroprevalence

Overall seroprevalence to combined *P. vivax* antigens (AMA+MSP) was 28% (732/2517), antibody responses to individual *P. vivax* antigens. In children with age less than or equal to five seroprevalence to combined *P. vivax* antigens (AMA+MSP) was 15% (113/732), while in older individuals (>20) it was 58% (424/732). Of all anti-*vivax* seropositive children of under five age group, 49% (55/113) were belonging to camp 3, where the lowest seroprevalence i.e., 6% (6/113) in children under five was observed in camp1. The seroprevalence data was in line with the infection data (RDT and PCR), which also shows camp 3 with high prevalence of *vivax* malaria as compared to other studied camps.

In this study the number of children with ≥ 5 ages having titres greater than 300 was 35 and 57 in case of anti-PvMSP and anti-PvAMA respectively, suggesting that these children may have had a recent infection or have hypnozoites. The serological data presented here provided strong evidence that there is ongoing local *P. vivax* transmission due to seropositive children under age five. As in previous studies conducted in low endemic areas, such as Costa Rica and Somaliland, strong antibodies responses in children with age less than five (indirect fluorescent antibody titres ≥ 20 in Costa Rica and ELISA antibody titres >200 to falciparum in Somaliland) were used as an evidence for active transmission of malaria (Bousema et al., 2010c, Warren et al., 1976, Warren et al., 1975). The data also suggest that there must have been *vivax* malaria transmission and exposure in the last 5 years due to high antibody responses in older age group individuals. This data is in agreement with BHU data of the camps. By comparing both antigens, seroprevalence to PvAMA-1 was high than to PvMSP-1₁₉, which is consistent with other studies (Drakeley et al., 2005, Stowers et al., 2002, Polley et al., 2004).

4.4.1.2.2. Age seroprevalence and seroconversion rates

The age stratified curves for combined *P. vivax* antigens (AMA+MSP) showed a clear pattern of increased sero-prevalence with age for all camps except camp 5. In camp 5 there is seen a uniform seropositivity across the population. The high proportion of *P. vivax* seropositive individuals in younger age groups made the data difficult to fit to the

conventional seroconversion model in camp 5. This indistinct pattern of age seroprevalence for camp 5 is because of the PvMSP-1₁₉ data. A similar unclear age seroprevalence profile was also observed for *P. vivax* anti-MSP-1₁₉ for camp 3 (see appendix 8). The ELISA assay was repeated to confirm the reproducibility of the results and double check to validate the data but the results were the same as previously observed.

Such unclear age seroprevalence profile, when all ages of the population are equally exposed to malaria for a defined period, are thought to be the result of an epidemic in an area (Cook, 2010). An alternative explanation for the observed high seropositivity in young children in camp 3 and 5 may be the presence of antibodies cross-reacting with antigens from other infectious agents such as leishmaniasis (cases are reported in this camp previously) or toxoplasmosis (Naus et al., 2003, Abramo et al., 1995).

Moreover the prevalence of infection detected by PCR among individuals aged 1-20 years was higher in camps 3 and 5 as compared to other camps (number of positives in 1-20 age group out of total positives was 62/79 and 17/23 in camp 3 and 5 respectively). And prevalence of infection (RDT and RDT) also showed a significant association with PvMSP-1₁₉ as compared to PvAMA-1 (Pearson's $\chi^2=0.9$, $P=0.4$ for AMA and for MSP $\chi^2= 47$ $P<0.001$), which means that PvMSP-1₁₉ positive individuals were more likely to be parasite positive. This is suggesting that PvMSP-1₁₉ might be the marker of recent infection and PvAMA-1 more of a cumulative marker of exposure in the study area.

However, according to previous studies, PfAMA-1 reflects recent exposure and antibodies against PfMSP-1₁₉ saturate over longer periods of exposure representing cumulative risks (Drakeley et al., 2005, Stowers et al., 2002, Polley et al., 2004). On the contrary, results from this study suggesting that parasite positive individuals are more likely to have antibodies to PvMSP-1₁₉ in the studied population rather than PvAMA-1. Although, the influence of hypnozoites in *P. vivax* infections (i.e. relapses) on serological profiles is also unknown (Bockarie and Dagoro, 2006).

Visual assessment of the age seroprevalence plots of combined antigens against *P. vivax* for camp 4 showed a poor fit of the model, for the age groups between 10 and 20, where seroprevalence was lower than predicted, suggesting a change in

transmission at this point. Such outliers in the seroprevalence curves may also be due to non-exposure of older people (In malaria free pre-war times in Afghanistan due intensive malaria control before Russian invasion in Afghanistan in 1978) and an age-dependent immune responses may not be primed if exposure is in later life.

Age seroprevalence curves for combined antibody responses to *P. vivax* antigens (AMA+ MSP) give better fits than looking at individual antibody responses to PvAMA-1 or PvMSP-1₁₉, which are given in appendix 7. PvAMA-1 showed a clear increase of seroprevalence with age as compared to PvMSP-1₁₉. But when examining the *P. vivax* MSP-1₁₉ antibody titres, there is seen a little increases with age, except in camp 3 the titre was high in children (age group 1-5) as compared to older age group (>20).

Age-specific sero-prevalence rates were used to estimate SCR which is an equal probability of a seronegative individual to become seropositive in a given interval of time at a rate λ year⁻¹, with the probability being a function of the immunogenicity of the antigen and the likelihood of being infected (Corran et al., 2007). A seroconversion rate (SCR) was calculated using a reversible catalytic model for each of the five Afghan refugee camps. The combined camps *P. vivax* seroconversion rate (λ) was estimated approximately 0.03. Our estimates of age-specific seroprevalence rates (SCR) of *P. vivax* demonstrate that there is heterogeneity in transmission across the studied camps, with camp 3 having high and camp1 have lowest SCR value, ranged from 0.01-0.07. Which are approximately equivalent to less than 0.12 to more than 3 infectious bites per person per year (EIR) for individual *P. falciparum* antigen. These estimation of EIR is based on the previously published survey (Corran et al., 2007), where SCR values were used to derive an estimated EIR values.

4.4.2. *Plasmodium falciparum*

4.4.2.1. Prevalence of infection measured by RDT and PCR

Overall *P. falciparum* parasite prevalence was very low; 0.04 % by RDT with only one *P. falciparum* infection positive individual in camp 5. Infection prevalence was 0% by PCR in three camps, with only one positive individual (0.7%) in camp 4 and 1.3% in camp 3 (7 positives). Of the 8 PCR positive individuals, 4 were children under five (Table 4). So whilst these results suggest a very low prevalence of *P. falciparum* but suggesting ongoing transmission.

The prevalence of *P. falciparum* infection in adults (age>30), suggests carriage of submicroscopic infection. As *P. falciparum* infections are associated with high morbidity but these positive individuals had no history of fever (fever history was collected during survey), these may represent submicroscopic asymptomatic infections.

The infections in children under five are perplexing as dogma suggests that these children would have little immunity and any infection would lead to illness with fever and/or infection detected by RDT. There are several possible explanations for these observations; firstly these results may be false positive due to PCR contamination. However, the positive samples were not adjacent or on different PCR plates suggesting no systematic contamination. Secondly, a recent work on submicroscopic malaria infections in different settings, suggested that the prevalence of submicroscopic infections in children and in low-transmission areas reflected that parasite-suppressing immune responses may develop more efficiently and infections may be older and more likely attained a submicroscopic phase as compared to infections in high transmission areas (Okell et al., 2012).

Overall, however the data presented here are consistent with the decline in disease burden of falciparum malaria in the last decade documented previously for the studied Afghan refugee camps in KP by BHU of the 2004-2009 (see appendix 14). Although direct comparison to previous BHU data is considerably limited as the data is passive or clinical data (the annual number of cases recorded by Afghan refugee BHU) and the present study was active surveillance. Previous studies in Afghan refugee camps in KP also has shown that *P. falciparum* more successfully reduced by interventions (IRS/ITN) than *P. vivax*, due to limited effects interventions have on relapses (Rowland et al., 1997a).

The survey was conducted before the transmission season (June-September) of falciparum malaria, so this might be another reason of zero % or extremely low (in two camps by PCR) parasite prevalence, as falciparum malaria infections peaks later (October-November) in the year (Rowland and Nosten, 2001). The first new cases of falciparum malaria can be usually seen in late August, although BHU recorded cases in May and June. But the number of cases of falciparum malaria in the studied camps by

BHU ranged from 0-5 in the last 6 years (2004-2009), which is in agreement with low prevalence of falciparum infection observed by this study.

4.4.2.2. Serological Data

4.4.2.2.1. Seroprevalence

P. falciparum seroprevalence to PfAMA-1 and PfMSP-1₁₉ was 4.4% and 2% respectively (detailed in appendix 3). Antibody levels for Children under age five seropositive to PfAMA-1 where antibody titre ranging from 5-4308 indicates that there is likely to be a very low ongoing local falciparum transmission. However no children of age group 1-5 were found seropositive to PfMSP-1₁₉ except in camp 5 (antibody titre ranged from 63-1325), showing no ongoing transmission (see appendix 3).

Overall seroprevalence to combined anti-falciparum antigens (PfAMA+PfMSP) observed was 6% (147/2513), but none of these individuals had clinical infection. Among the 147 individuals who were seropositive for *P. falciparum* antibodies (AMA+MSP), 30 (20%) were children under the age of five years suggestive of recent malaria exposure. Over 56% (17/30) of these children were from camp 5. Seroprevalence in the oldest age (>20) group was 49% out of the total 147 seropositive individuals.

In previous studies conducted in low endemic areas Costa Rica and Somaliland strong antibodies responses (indirect fluorescent antibody titres ≥ 20 in Costa Rica and ELISA antibody titres >200 to falciparum in Somaliland) were used as an evidence for active transmission of malaria (Bousema et al., 2010c, Warren et al., 1976, Warren et al., 1975). In our study the antibody level (titre) in children under five who were seropositive ranges from 6-4308. Seven out of thirty seropositive children of age ≥ 5 had high antibody titres ranges from 550-4308 to *P. falciparum*, thus presenting more evidence of recent infection. These titres are not directly comparable due to the differences in antigen used, different study populations and technique used to measure antibody levels (ELISA and IFAT) in the above-mentioned studies. However, these high levels of titre in children having age less than five suggesting that kids with high antibody titres are likely to be a result of recent infection, especially if positive to both antigens used against *P. falciparum*.

The study revealed extremely low levels of falciparum malaria detected by serology at all study sites which are consistent with the parasitological data of the study and available basic health unit records of malaria (low AFI from 2004-2009 appendix 14). Low seroprevalence was even observed in adults (>20 years), which suggests that may be *P. falciparum* transmission was never very high in the study area or in the group resident here. Low overall seroprevalence (6%) and with very little evidence of age specific increase in the seropositive individuals or titre is suggesting that there is no ongoing transmission. But due to presence of sub-RDT *P. falciparum* infection and few reported cases by BHU year before the survey (2009), suggesting that there is still very low ongoing transmission of *P. falciparum* in the camps.

It is widely considered that antibody responses require repeated exposure to become established at detectable level in low transmission areas. On the other hand may be due to low transmission intensity in the area, many individuals would be more likely to lose their anti-falciparum antibodies that were previously acquired before building up to detectable level. Or the IRS campaign that was in placed during 1990s may be one of the reasons to reduce exposure to malaria, or may be other malaria control programme (ITN) helped to control the level of falciparum malaria. Due to very low falciparum malaria transmission intensity in the study site and the presence of malaria vectors make it potentially more susceptible to malaria epidemics. For example several epidemics were recorded among refugees in the 1990's, over 150 000 cases were being diagnosed and treated each year by the combined health services of the UNHCR, the government of Pakistan and NGO (Rowland, 1999a, Rowland and Nosten, 2001). The number of cases has declined as control measure took effect (Rowland et al., 2002 b).

The *P. falciparum* seroprevalence showed evidence of heterogeneity with camp 5 showing the highest seroprevalence (11%) and camp 4 showing the lowest (3%) among the five camps. These regional differences were statistically significant with $p > 0.01$ (using Pearson's χ^2 test) for all *P. falciparum* antigens (PfAMA, PfMSP and combined anti-Pf antigens). Variability in the AFI among the camps was observed in the BHU data and also in serology data from this study.

4.4.2.2.2. Age seroprevalence and seroconversion rates

Because of extremely low overall seroprevalence of *P. falciparum* no clear increase of sero-prevalence with age was observed. Therefore age stratified curves for *P. falciparum* showed little increase in seroprevalence with age to combined antigens (AMA+MSP). Age seroprevalence curves for each of the *P. falciparum* antigens showing little increase of seroprevalence with age (AMA-1) to no increase (MSP-1₁₉) (see appendix 5 and 6 respectively). In areas where transmission has been consistently low for many years a loss in seropositivity in older individuals may result in uniform seropositivity across the population; masking any change point in transmission. Previous sero-epidemiology study conducted in Tana (Vanuatu) also suggested that in areas of very low transmission, models used to assess an increase in seropositivity with age may be inappropriate (Cook, 2010). In a situation where age seroprevalence reveals little information, investigation of antibody titres may be more useful (Cook et al., 2010). But using antibody titres to study an increase in seropositivity with age was also uninformative except for camps 1 and 2 using PfMSP-1₁₉ data as no clear pattern of increase of antibody titres with age for PfAMA-1 was observed.

Estimated transmission intensity based on SCR for *P. falciparum* varied very little from camp to camp, where camp 2 and 5 had high SCR values than the rest of the camps. The *P. falciparum* seroconversion rate (λ) for all the camps combined was estimated to be approximately 0.005. There is no obvious evidence of recent changes in transmission. In a similar transmission setting in Somaliland Bousema et al., described SCR of 0.005 for PfAMA-1 and 0.008 for PfMSP-1₁₉. These seroconversion rates were considered to be equivalent to an EIR of >0.1 using a validated model to relate age-specific seroconversion rate to EIR (Corran et al., 2007). In this study SCR value for PfAMA-1 is higher compared to PfMSP-1₁₉, which are 0.003 and 0.001 respectively.

4.4.3. *P. vivax* vs. *P. falciparum*

It is widely held in Pakistan that *P. falciparum* constitutes less than 30% of all malaria infections, and 70-90 % of malaria infections are caused by *P. vivax* in this study population (Rowland et al., 1997a). However, these data needed to be confirmed, as *P. vivax* infections are often missed during routine clinical microscopy. The data from this study confirmed that *P. vivax* is the predominant malaria in the area. This is similar to

areas of low transmission intensity where *P. vivax* prevalence is also found higher than *P. falciparum* (Noor et al., 2011). The possible reasons given for this difference in the prevalence of the two mentioned malaria species are:

Firstly, the presence of infected hypnozoites in liver in case of *P. vivax* which would not be detected by microscopy and difficult to clear these hypnozoites as G6PD deficiency makes the *P. vivax* control challenging. Monthly BHU malaria data from these Afghan refugee camps also showed that there is monthly fluctuation of *P. vivax* over the season but not of *P. falciparum*. The fluctuation of *P. vivax* seen throughout the year may be due to different life cycle of *P. vivax* in which hypnozoites remain in infected liver cells for long time and cause relapses months or years later. Secondly different susceptibility of malaria vectors may also be the reason for differences in transmission.

An increase in *P. vivax* cases has been also reported in areas where *P. falciparum* transmission appears to be on the decline (Noor et al., 2011, Stewart et al., 2009, Stowers et al., 2002). One possible explanation given is that immunity developed to one species of plasmodium affect the acquisition of immunity to the other species (Gamage-Mendis et al., 1991, Maitland et al., 1996). Some studies suggested that due to cross stimulation of immune response *falciparum* could be suppressed by the presence of *vivax* (Haghdoust and Alexander, 2007, Maitland et al., 1996, Nagao et al., 2008, Whitehorn et al., 2010). The high *P. vivax* seroprevalence seen in children under five may be due to the fact that clinical immunity is rapidly acquired for *P. vivax* than for *P. falciparum* (Maitland et al., 1996).

4.4.4. Association of serological, parasitological measures and EIR

Estimates of SCR showed a strong relationship with estimates of parasite rate of *P. vivax* (when detected by RDT) for individual antigens i.e. PvMSP-1₁₉ or PvAMA-1 and also when both antigens were combined (PvAMA+PvMSP) for *P. vivax* (Fig.4.11). The relationship was found very weak between PCR detected parasite rate and SCR when looking at the individual antigens, for AMA-1 and MSP-119 (Fig.4.11). But the relationship was observed moderate ($R^2=0.5$) between parasite rate detected by PCR and SCR (estimated from seroprevalence to combined antigens (AMA+MSP)) although with a greater degree of scatter (Fig.4.11). A strong relationship was also observed between SCR and parasite prevalence detected by microscopy (Cook, 2010). This

indicates the utility of the serological assay for detecting level of malaria endemicity in different study sites. The relationship between *P. falciparum* parasite rate and SCR could not be determined as the parasite rate was 0% and was extremely low when PCR based detection was used.

The resulted EIR values for the camps may not be as accurate as was calculated for Tanzanian data but may be indicative of EIR in the study sites. Firstly due to unavailability of the model based on *P. vivax* SCR values to estimate EIR. Secondly as mentioned above the estimation of EIR was made on the basis of using single *P. falciparum* antigen PfMSP-1₁₉ and I estimated SCR by combining antibody responses to both *P. vivax* antigens (estimated SCR values for individual *P. vivax* antigen are given in appendix 10). Moreover, *Plasmodium vivax*, a species with relapses and as serology do not “count” the number of relapses which may also lead to overestimation of EIR using seroconversion rates for *P. vivax*.

Approximately 59.4% of the *P. vivax* antibody positive individuals were parasite positive detected by RDT and 50% of individuals were both seropositive and parasite positive when detected by PCR. The sensitivity of serological measure to detect parasite positive person is higher when RDT is used compared to PCR and may reflect that RDT detects higher density of parasite than PCR. It may be more likely about the fact that the PCR positive individuals have lower parasite densities and these may not have induced detectable immune response that is detected by PCR.

The sensitivity of available *P. vivax* antigens to detect parasite positive individuals is less than the *P. falciparum* according to some previous studies. For example a study carried out in Cambodia have shown only 42% sensitivity of *P. vivax* antigens to detect currently *P. vivax* infected individual and *P. falciparum* showed 92% sensitivity (Cook, 2010). While in Korea when five antigens (circumsporozoite protein (CSP-1), merozoite surface protein (MSP-1), apical merozoite antigen (AMA-1), serine repeat antigen (SERA), and exported antigen (EXP-1)) were combined sensitivity of *P. vivax* antigens was increased to 80%, as using one antigen was not sufficient to detect infection of *P. vivax* (Kim et al., 2003). However according to a study conducted in Brazil the sensitivity was 90% (Rodrigues et al., 2003). In Iran (bordering Pakistan),

which is also *P. vivax* dominant area, the sensitivity was recorded 81% using PvAMA-1 (Haghi et al., 2012).

Possible explanations for the lack of reactivity observed in some of the parasite positive individuals to the *P. vivax* antigens in the above mentioned studies are, firstly, there may be comparatively more allelic polymorphism in *P. vivax* strains than there are in their *P. falciparum* homologues. So possible the antigens used here were not suitable for this population. A Brazilian study however, showed that the DNA sequences encoding MSP-1₁₉ were conserved in *P. vivax* isolates as compared to the equivalent region of other species of Plasmodium. Amino acid sequences of PvMSP-1₁₉ from Southeast Asian and Latin American strains showed only one amino acid substitution (Pasay et al., 1995). However, it is unknown whether this single substitution is of immunological significance (Soares et al., 1999). Previous studies have also shown that there may be a good response of antibodies to different strains of *P. vivax*, as antibodies are directed mainly to conserve epitopes present in both allelic forms of the protein (Soares et al., 1999). The gene coding for AMA-1 is highly conserved among Plasmodium spp. of rodents, monkeys, and humans. However, intra-species unlinked single base substitutions are observed in some regions of the gene (Figtree et al., 2000). Secondly, may be many individuals have few infections of *P. vivax* as compared to *P. falciparum* as specific antibodies do not build up to a detectable level. Alternatively, the parasite positive individuals who did not respond to PvAMA-1 and PvMSP-1₁₉ may produce antibodies to different antigens of the parasite (Cook, 2010).

Sensitivity of the assay was low to detect parasite positive assay in this study, which can be increased further by inclusion more antigens in the future in the study area. Both serological and parasitological data revealed low levels of vivax and extremely low level of falciparum malaria, therefore carefully planned control programmes can further reduce transmission in the these camps. Furthermore, continual surveillance and monitoring interventions can finally lead control to elimination.

On the other hand reduction seen in the antibody levels especially in case of falciparum malaria symbolising reduction (loss) in the clinical immunity. Several reviews have discussed the possible devastating effects of reducing exposure to

transmission in young ages and on population immunity levels (Askjaer et al., 2001, Ghani et al., 2009). However, a study on submicroscopic infection challenged the common assumption that individuals with little previous exposure to malaria have a limited immune response against parasites (Okell et al., 2012). The study suggested that prevalence of submicroscopic infections in the individuals with little past exposure to malaria develops parasite-suppressing immune response more efficiently than commonly assumed (Okell et al., 2012). However, whether these low levels of antibody responses will result to reduction in the clinical immunity still remains to be seen in the study area.

On the other hand, low malaria transmission intensity detected by this study and the presence of malaria vectors may increase chances of epidemics of malaria, as recorded previously in the Afghan refugee camps in Pakistan (Rowland and Nosten, 2001). Such epidemics were thought to result in a high mortality rate in resource-poor areas like the studied camps (Guthmann et al., 2007), especially when there are no outbreak detection systems (Coleman et al., 2008).

Low levels of parasitemia detectable only by PCR are thought to be able to restart transmission in favourable conditions. For example in Sudan, during the long dry season prevalence of infection detected by microscopy were reported zero but some PCR-positive cases result in resurgence of transmission in the rainy season (Roper et al., 1996). Therefore, for successful control, survey of the prevalence of asymptomatic cases in the camps is vital in many malaria settings (Karl et al., 2011). It is also assumed that the prevalence of submicroscopic infections in an area may reflect recent transmission dynamics of malaria, that a high prevalence of submicroscopic infections may suggest a recent decrease in transmission (Gatton and Cheng, 2010).

For effective control and elimination of malaria, determination of its transmission and burden is obviously crucial. Although, identification of possible risk factors of malaria and assessment of its relationship with infection and exposure can benefit further prevention and control strategies in the area. These are discussed in detail in the next chapter with spatial analysis of malaria infection and exposure to identify hot spots of malaria within the camps for more focal malaria control.

To identify behavioural and residential risk factors of malaria exposure and infection in the refugee camps

Chapter 5. To identify behavioural and residential risk factors of malaria exposure and infection in the studied camps

The results presented in this chapter are from the household survey described in previous chapters. This is the first ever malaria prevalence and risk factor survey based on individual and household characteristics in this area. The aim of this study was to identify possible risk factors of malaria and to assess its relationship with infection and exposure to malaria in order to benefit prevention and control strategies in the area. The study mainly focused on individual characteristics (fever history, traveling history and protection from mosquitoes) and the household characteristics included household construction material, roof, floor, eaves, windows, toilet facilities, and socio economic status as potential risk factors. The relationship of individual risk and household characteristics to the number of malaria infections and seroprevalence were examined. The study also assessed the coverage of interventions such as net ownership, ITN use and spraying of the household in last 12 months with insecticide.

As previously stated KP is characterized as a low malaria endemic area (Bouma et al., 1996a, Rowland et al., 1997a), where malaria is seasonal and the majority of transmission occurs after monsoon (July-August). The prevalent malaria vectors are *Anopheles stephensi* and *Anopheles culicifacies*, which mainly breed in river pools, river edges and irrigated fields. Monsoon rainfall offers additional larval habitats, which have the potential to increase malaria transmission. The Afghan refugee camps are commonly situated on marginal land, many of them are waterlogged or adjacent to rice cultivation, rivers and streams, therefore proximal to mosquito breeding sites (Rowland and Nosten, 2001).

Afghan refugees were provided with tents when they first arrived in Pakistan after the Russian invasion in 1978. Soon refugees constructed their homes and walled compounds from mud and stone in the local Pakistani villages style (Christensen, 1998). Mud houses, thatched roof, non glazed windows and presence of eaves in the living rooms are a poor barrier to vector mosquitoes. In the hotter humid summer months (transmission season of malaria) most of people prefer to sleep outdoors in

the courtyard which most likely to increase man–vector contact(Rowland et al., 2002 b).

Malaria transmission and prevalence are determined by local ecological conditions, which can vary from village to village (Ye et al., 2007, Ramachandran, 2010). Previous studies conducted to identify the “local” risk factors for malaria transmission investigated travel history (cases can be introduced from other areas), (Moore et al., 2004, Osorio et al., 2004, Joshi et al., 2006), proximity to water bodies (Bousema et al., 2010c), forest malaria (Cook, 2010), and socio-economic conditions (Ramachandran, 2010).

Malaria has always been most endemic in rural areas and a major challenge to control compared with urban areas worldwide. For successful control and elimination of malaria at community level, it is important to take into account human behaviour, socio cultural and economic context through active participation and changing of risk behaviour of the community (Williams and Jones, 2004, Opiyo et al., 2007). This study aims to assist control programmes by providing information about socio-economic and behavioural risk factors of malaria in the study area.

5.1. Material and methods

5.1.1. Laboratory method

ELISA was performed as described in chapter 2 and antibody responses were detected against antigens from both *P. falciparum* and *P. vivax*. PCR for malaria parasite species detection and for detection of sub-RDT infections was also conducted.

5.1.2. Statistical analysis

The data analysis for the study was done using STATA version 12 using the survey command (svy) with camps as the strata and household as the primary sampling unit. 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. The outcome (response) variable was the presence or absence of malaria using the RDT, PCR and combined antibody responses to *P.falciparum* (response to either PfAMA-1 or PfMSP-1₁₉) and *P. vivax* (response to either PvAMA-1 or PvMSP-1₁₉). Potential risk factors tested in the models were: age in years, sex, travel history, malaria fever history, house construction (roofing material, wall material, floor material, presence of eaves and window glazing), recent or regular bed net use, insecticide residual spray, 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 (prevalence of infection and seroprevalence) 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 $p \leq 0.05$.

5.1.2.1. Measurement of socio-economic status

Principle component analysis (PCA) is a multivariate statistical 'data reduction' procedure used to reduce a set of correlated variables in a data set into a set of uncorrelated smaller 'principal components' which represent unobserved characteristics (Vyas and Kumaranayake, 2006, Howe et al., 2008).

PCA can be represented mathematically as:

For example, from a set of variables X_1 through to X_n ,

$$PC1 = a_{11}X_1 + a_{12}X_2 + \dots + a_{1n}X_n$$

$$PC2 = a_{m1}X_1 + a_{m2}X_2 + \dots + a_{mn}X_n$$

where a_{mn} represents the weight for the m th principal component and the n th variable (Howe et al., 2008).

Principal component analysis (PCA) was used on household asset ownership data to measure socio-economic status in this study. Used guideline for PCA for wealth indices were published elsewhere (Vyas and Kumaranayake, 2006, Armstrong Schellenberg et al., 2008, Howe et al., 2008).

Household assets ownership data was analysed using Stata (version 12, College Station, Texas, USA) to create household wealth index. The Socio economic status of household was the weighted sum of household characteristics including whether they owned the consumer durables such as a television (Tv), fridge, radio, bicycle, mobile, animals, poultry and whether they used wood for cooking, whether they had mains electricity, the type of house construction, roof (iron roof/thatched roof), floor (cemented/mud) and toilet. Principal Components Analysis (PCA) on the correlation matrix generated weights for the assets in the index. Households were categorized into one of five equal sized groups from the most poor to the least poor (Armstrong Schellenberg et al., 2008). Summarized data was presented in Table 5.2.

Table 5.1 Assets ownership for households in each socio-economic status quintile

Wealth quintile						
Percentage(number) of households having assets	1(Most poor)	2(very poor)	3(Poor)	4(less poor)	5(least poor)	Total
Households	22.3%(188)	16.6%(140)	17.8%(150)	20.9%(176)	22.5%(190)	844
Tv	0.8(2)	0.8(2)	3.8(10)	29.9(78)	64.8(169)	30.9(261)
Fridge	0	0	2.4(6)	27.3(68)	70.8(175)	29.5(249)
Radio	2.9(18)	21.4(133)	19.3 (120)	26.5(165)	29.9(186)	73.7(622)
Bicycle	9.7(69)	19.5(138)	20.5(145)	24.4(173)	26(184)	84(709)
Mobile	18.4(146)	17.4(138)	18.9(150)	21.8(173)	23.7(188)	94.2(795)
Electricity	22.2(187)	16.6(140)	17.8(150)	20.9(176)	24.5(892)	99.8(842)
Chickens	21.6(175)	15.6(126)	18.5(149)	21.3(172)	23.1(187)	95.9(809)
Animals ownership	17.4(71)	1.2(5)	30.6(125)	23.2(95)	27.6(113)	48.5(409)
Fuel (electricity/gas)	0	0	33.3(3)	22.2(2)	44.4(4)	1.1(9)
House construction	3.5(2)	5.7(3)	5.2(3)	10.3(6)	75.9(44)	6.9(58)
Toilet(flush system)	1(2)	0.5(1)	2.8(6)	24.2(51)	71.6(151)	25(211)
Floor	3.1(2)	3.1(2)	7.7(5)	15.4(10)	70.7(46)	7.7(65)
Roof	1.2(3)	0	5.8(14)	22.6(55)	70.4(171)	28.8(243)

The table is showing the number and percentages of household assets from the most poor to the least poor quintile. Household characteristics/assets including ownership of Television (Tv), fridge, radio, bicycle, mobile, animals, poultry and type of fuel used for cooking (wood, dung, kerosene oil or electricity/gas) whether they had mains electricity, the type of house construction (brick and stones or mud, roof (iron roof/ thatched roof), floor (cemented/mud) and having flush toilet system.

5.2. Results

The total number of households surveyed was 845 in 5 camps. Overall 2526 individuals were examined for recent malaria infection by RDT and blood samples were taken on filter paper for molecular diagnosis of malaria and serological analysis. Data about fever history, travelling history, ITN use and personal mosquito protection was collected from each study individual, no missing data was found except for the ELISA data, which was due to discordant ODs and applied to a total of 11 samples which were discarded.

Table 5.2Detail and division of potential risk factors for malaria in the camps

Individual and Behavioral risk factors	Household/residential risk factors	Vector control
Age	House construction	Insecticide residual spray (IRS)
Sex	Roof	Insecticide treated bed nets (ITN)
Travelling history	Eaves	ITN Used last night before survey
Fever history	Windows glazing	Personal Mosquitoes Protection
Treatment history	socio-economic status (SES)	
	Animals ownership	

The factors in the Table 5.2 were included in the study to see its effect on the infection prevalence and seroprevalence of malaria. Individual or behavioral risk factors included age (1-5, 6-20 and above 20 age groups), sex, fever history (fever in last 24 hours or two weeks prior to survey), treatment history (sought treatment or not, treatment centre including BHU/govt.hospital/private clinic/pharmacy/field worker, antimalarial taken in last two weeks before survey), travelling history (travelling outside of district in last 3 months before the survey, returned from recent trip including returned within two or four weeks) or ITN used last night prior to survey. Household risk factors were comprised of household construction (house build of bricks and stones or mud), roof material (iron rods or thatched grass), Windows glazing, socio-economic status by quintile (SES) and Animals ownership. While vector control measures included to study were, IRS (IRS within 12 months), ITN ownership and personal protection from mosquitoes (use of mosquitoes coil, can spray or repellents).

Demographic and vector control measures information

All infections of malaria detected by RDT were *Plasmodium vivax* with 4% prevalence (96/2522). PCR was done on selected number of samples, which detected 12% of infection positive individuals (140/1051). Majority of *P. vivax* infections were found in females 60.7% (85/140) when measured by PCR, but there were only marginal differences of *P. vivax* infections between male (49/96) and female (47/96) when measured by RDT. Seroprevalence was higher in females than in males to both *P. vivax* (66%) and *P. falciparum* (58%). Data collected on the use of malaria vector control measures is shown in Table 5.3, where 48.6% of individuals reported having ITN in the camps with 28.6% people having used ITN the night before the survey.

Table 5.3 Vector control measures used in refugee camps

Camp	Adezai (1) %	Baghicha (2) %	Jalala (3) %	Kagan (4) %	Zangal patai (5) %	Total %
IRS within 12 months	4.2	2.4	7.8	2.4	10.5	5.4
ITN Ownership	32	78.7	47.6	65.9	20	48.6
ITN Used Last Night	16	63.7	3.2	51.4	9.3	28.6
Personal Protection Measures used against vector						
NO	19.5	47.3	68.9	50.6	56.2	48.5
Coil	76.3	52	30.5	46.3	39	48.9
Insecticide spray (can)	2.4	1	0	0.6	2.4	1.2
Repellents	1.8	0	1	2.4	2.4	1.4

The table is showing percentages of households who plot IRS in the last 12 months prior to survey, owned ITN, use of ITN previous night before survey and vector control measures used for personal protection which included mosquitoes coils/spray cans/repellents.

5.2.1. Risk factors of malaria in study area

5.2.1.1. *P.vivax* infection and risk factors

5.2.1.1.1. Univariate Analysis

A full list of factors investigated for association with the prevalence of *P. vivax* by RDT and PCR are shown in Tables 5.4 and 5.5 respectively.

Individual or behavioral risk factors

The prevalence of *P. vivax* infection detected by RDT decreased with increasing age (in age group >20 the OR= 0.4, $p<0.01$). Women were less likely to be parasite positive than men when infection measured by RDT (RDT, OR= 0.6, $p= 0.05$) but the difference was not significant ($p= 0.80$) when PCR prevalence was used as an outcome variable. Individuals with fever history (fever within 2 weeks or 24 hours) were strongly associated with parasite prevalence (RDT, OR= 6.4, $p<0.01$; PCR OR= 4.6, $p<0.01$ for fever within 2 weeks and RDT, OR= 13.9, $p<0.01$; PCR OR= 6.8, $p<0.01$ for fever within 24 hours).

Individuals who reported having sought malaria treatment in the past had significantly higher prevalence of infection when detected by RDT but not by PCR (RDT, OR= 2.5, $P= 0.024$; PCR, OR= 2.1 with $p=0.067$). Administration of antimalarial drugs in the past two weeks before the survey was not significantly associated with prevalence of infection measured by either RDT or PCR.

Prevalence of *P. vivax* was lower in individuals who had remained in the camp of resident for the last six months (RDT, OR= 0.1, $p< 0.02$; PCR, OR= 0.2, $p< 0.01$), while outside travel within 3 months from the resident camp and returning from recent trip were not significantly associated with increased prevalence.

Risk factors related with household

Individuals living in a household built of bricks were less likely to be parasite positive (PCR, OR= 0.3, $p<0.05$). Household construction type (mud/brick) was not significantly associated with RDT positivity. While roof constructed of iron roof and sheets was a significantly associated with lower risk of *P. vivax* infection when detected by RDT, OR=

0.4, $p < 0.01$; but was non-significant when detected by PCR (Table 5.4). Individuals living in a room with no eaves were most likely *P. vivax* RDT negative (RDT, OR= 0.4, $p < 0.01$; PCR OR= 0.5, $p < 0.01$) than the individuals sleeping in room having eaves (Table 5.4). Window glazing was not significant risk factor of malaria in the studied camps by both measures. In univariate regression analysis the prevalence of *P. vivax* infection by both RDT and PCR decreased if individuals were from the least poor households SES bracket compared to those from the poorer households (RDT, OR= 0.2, $p < 0.01$; PCR OR= 0.3, $p < 0.01$) (Table 5.4).

Factors related to limit and control vector

The use of ITN on the night prior to survey was associated with protection from *P. vivax* infection (RDT, OR= 0.2, $p < 0.01$; PCR OR= 0.5, $p < 0.02$). ITN ownership was not significantly associated with RDT prevalence of *P. vivax* infection. ITN in the house significantly associated with increased risk of *P. vivax* infection when detected by PCR (OR= 1.6, $p < 0.03$) (Table 5.4). Using of mosquitoes coils/spray can or repellents for protection from mosquitoes were significantly associated with the decreased prevalence of *P. vivax* when infection was measured by RDT (OR= 0.2, $p < 0.01$) but the association was not significant when detected by PCR. IRS within 12 months was not significant between those with and those without vivax infection.

5.2.1.1.2. Mul variate variate Analysis

The details of multivariate logistic regression analysis for risk factors and prevalence of vivax infection detected by RDT and PCR are given in Tables 5.4 and 5.5 respectively. The multivariate regression model showed a significant association of *P. vivax* infection (detected by both RDT and PCR) and stay in the camp of residence since last 6 months before the survey, previous and current fever status (fever within 24 hours/fever within two weeks) and last night ITN use age of individual. SES of household was a significant risk factor of vivax parasite prevalence in the camps only when infection detected by PCR, while sought malaria treatment and use of mosquito coils/repellents were significantly associated with the vivax infection when detected by RDT.

Table 5.4 Univariate and mul variate analysis for prevalence of *P. vivax* infec on diagnosed by RDT

<i>P. vivax</i> (RDT)	Univariate		Mul variate	
	OR	p<	OR	p<
Risk factors				
Age groups				
1-5	1		1	
6-20	0.8(0.5-1.3)	0.35	0.8(0.5-1.2)	0.25
>20	0.4(0.2-0.7)	0.01	0.3(0.2-0.6)	0.01
Sex				
Male	1			
Female	0.6(0.4-1.0)	0.05		
Stay last 6 months in the camp				
No	1			
Yes	0.1(0.0-0.7)	0.01	0.2(0.04-0.7)	0.02
Fever in the previous 24 hours				
No	1			
Yes	13.9(5.7-34.3)	0.01	10.6(3.1-36.8)	0.01
Fever in the previous 2 weeks				
No	1			0.01
Yes	6.4(3.61-11.3)	0.01	47.6(15.3-147.9)	
Sought malaria treatment before				
No	1			
Yes	2.5(1.1-5.5)	0.02	0.1(0.02-0.2)	0.01
Roof				
Thatched grass	1			
Iron sheets and rods	0.4(0.2-0.7)	0.01		
Eaves				
Open	1			
Closed	0.4(0.2-0.7)	0.01		
SES (Most to least poor)				
1	1			
2	0.4(0.2-0.9)	0.03		
3	0.6(0.3-1.3)	0.17		
4	0.5(0.2-1.0)	0.04		
5	0.2(0.1-0.3)	0.01		
ITN last night				
No	1			
Yes	0.2(0.1-0.5)	0.01	0.1(0.1-0.5)	0.01
Using coil/repellents/spray can				
No	1			
Yes	0.3(0.2-0.5)	0.01	0.3(0.2-0.6)	0.01

Table 5.5 Univariate and mul variate model for prevalence of *P. vivax* infec on detected by PCR

<i>P. vivax</i> (PCR)	Univariate		Mul variate	
Risk factors	OR	p<	OR	p<
Age groups				
1-5	1			
6-20	1.3(0.8-2.2)	0.25		
>20	1.0(0.6-1.7)	0.90		
Stay last 6 months				
No	1		1	
Yes	0.2(0.1-0.6)	0.01	0.4(0.2-0.9)	0.03
Fever in the previous 2 weeks				
No	1		1	
Yes	4.6(2.5-8.3)	0.01	3.5(1.8-7.0)	0.01
Fever in the previous 24 hrs				
No	1		1	
Yes	6.8(3.0-15.1)	0.01	4.6(1.8-11.6)	0.01
Household construc on				
Mud	1			
Bricks/Stones	0.3(0.1-1.0)	0.04		
Eaves				
Open	1			
Closed	0.5(0.3-0.9)	0.01		
SES (Most to least poor)				
1	1		1	
2	0.6(0.3-1.2)	0.16	0.6(0.3-1.3)	0.21
3	0.5(0.3-1.0)	0.04	0.6(0.3-1.2)	0.23
4	0.6(0.4-1.1)	0.08	0.5(0.3-1.0)	0.07
5	0.3(0.2-0.6)	0.01	0.3(0.2-0.7)	0.01
ITN ownership				
No	1		1	
Yes	1.6 (1-2.3)	0.04	2.4(1.5-3.8)	0.01
ITN last night				
No	1		1	
Yes	0.5(0.2-0.9)	0.02	0.2(0.1-0.6)	0.01

5.2.1.2. Risk factors for *P.vivax* exposure

5.2.1.2.1. Univariate Analysis

Initial univariate analysis (weighted for sampling probability and adjusted for clustering by using svy command in Stata) identified the following factors associated with antibodies responses to vivax malaria (Table 5.6).

Individual or behavioral risk factors

Being in an older age group i.e. >20 (OR=7.3, 95% CI 5.6-9.4, $p<0.01$) and females (OR=1.7, 95% CI 1.3-2.0, $p<0.01$) were strongly associated with the likelihood of being seropositive to *P. vivax*. Individuals who travelled outside of the resident camp within 3 months (OR=1.9, 95% CI 1.5-2.5, $p<0.01$) and ones who returned back to camp from travelling within 2 or less than 4 weeks were more likely seropositive (OR=2.4, 95% CI 1.8-3.3, $p<0.01$ and OR=1.8, 95% CI 1.1-2.9, $p<0.02$ respectively). Fever within two weeks before survey (OR=3.2, 95% CI 2.2-4.6, $p<0.01$), individuals who sought malaria treatment after having malaria infection (OR=3.5, 95% CI 2.3-5.2, $p<0.01$), and individuals who taken antimalarial drugs within last two weeks before survey (OR=2.9, 95% CI 1.9-4.5, $p<0.01$) were significantly associated with increased *P.vivax* seroprevalence.

The analysis showed no association between *P. vivax* seroprevalence and individuals who had remained in the camp of resident from the last six months and fever within 24 hours prior to survey.

Risk factors related with household

In the residents of households; having eaves in living room (OR=3.4, 95% CI 1.5-7.8, $p<0.01$) and non glazed windows (OR=7.3, 95% CI 5.6-9.4, $p<0.01$) were associated with significant increase in seroprevalence. Individuals sleeping with animals in the same courtyard (OR=1.6, 95% CI 1.3-1.9, $p<0.01$) were also found with increased risk of *P. vivax* seroprevalence. The analysis showed no association between *P.vivax* seropositivity and roof material, household construction and SES.

Factors related to limit and control vector

Use of mosquito coils, spray cans or repellents (collectively these are personal protective measures against malaria vector) were strongly associated with reduced antibody responses to *P. vivax* (OR=0.6, 95% CI 0.5-0.8, $p<0.01$) (Table 5.6). There was a trend that house spraying within the previous 12 months and sleeping under a net previous night was associated with reduced or lower seroprevalence, but this relationship was not statistically significant in this study using *P. vivax* antibody responses as an outcome variable (IRS, OR=.8 with $p<0.4$ and ITN, OR=0.9 with $p<0.23$).

5.2.1.2.2. Mul variate variate Analysis

The final multivariate model identified increasing age (age group > 20 with OR=7.9, $p<0.01$), fever within 2 weeks (OR=1.7, 95% CI= 1.3-2.1, $p<0.01$) and sleeping with animals in the same house (OR= 1.6, 95% CI= 1.3-2.1, $p<0.01$) and Individuals who were not using personal protection against mosquitoes (coils, spray cans and repellents) (OR=0.6, 95% CI= 0.5-0.8, $p<0.01$) were associated with increased risk of being seropositive against *P. vivax* antigens. The detail is given in Table 5.6.

Table 5.6 univariate and mul variate analysis *P.vivax* seroposi vity

<i>P.vivax</i> (Seroposi ve)	OR (Univariate)	P value	OR(Mul variate)	P value
Age groups				
1-5	1		1	
6-20	1.9(1.4-2.5)	0.01	1.9(1.4-2.5)	0.01
>20	7.3(5.6-9.4)	0.01	7.8(5.8-10.5)	0.01
Sex				
Male	1			
Female	1.7(1.3-2.0)	0.01		
Outside travel within 3 months				
No	1			
Yes	1.9(1.5-2.5)	0.01		
Return recent trip				
No trip	1			
<2 weeks	2.4(1.8-3.3)	0.01		
< 4 weeks	1.8(1.1-2.9)	0.02		
> 4 weeks	1.1(0.7-1.8)	0.7		
Fever in the previous 2 weeks				
No	1			
Yes	3.2(2.2-4.6)	0.01	2.1(1.0-4.7)	0.05
Sought malaria treatment before				
No	1			
Yes	3.5(2.3-5.2)	0.01		
An malarial drug taken within last 2 weeks				
No	1			
Yes	2.9(1.9-4.5)	0.01		
Sleeping in the same courtyard with Animals				
No	1		1	
Yes	1.6(1.3-1.9)	0.01	1.6(1.3-2.1)	0.01
Eaves in living rooms				
Open	1			
Closed	0.8(0.6-1.0)	0.05		
Windows glazed				
No	1			
Yes	0.8(0.6-1.0)	0.03		
Using coil/repellents/spray can				
No	1		1	
Yes	0.6(0.5-0.8)	0.01	0.7(0.5-0.8)	0.01

5.2.1.3. *P. falciparum* and risk factors

5.2.1.3.1. Analysis of risk factors and *P. falciparum* infection

Detailed statistical risk factors analysis for *P. falciparum* infection was not performed as parasite prevalence by RDT was zero in four of the five the camps except for one positive sample in Zangal patai camp (camp 5). Overall prevalence of *P. falciparum* infection measured by PCR was 0.76% (8/1051) with 7/505 in Jalala camp (camp 4) and 1/498 in Kagan camp (camp 4) (See Chapter 4 results).

5.2.1.3.2. Risk factors for *P. falciparum* exposure

Seroprevalence against *P. falciparum* combined antigens (AMA-1+MSP-119) was 6% (147/25130). To identify potential risk factors for *P. falciparum* seroprevalence a regression model was developed using *P. falciparum* seroprevalence as the outcome. All the detail of significant variables of univariate and multivariate analysis is given in Table 5.7.

5.2.1.3.3. Univariate Analysis

Individual or behavioral risk factors

In the univariate logistic regression analysis model, individuals in age group of >20 were more likely to be *P. falciparum* seropositive than the children equal to or less than five years age (OR = 3.1, 95% CI 1.8-4.9, $p < 0.01$). Stay in the camp of residence from the last six months prior to the survey was significantly associated with reduction in seroprevalence to *P. falciparum* (OR=0.1, 95% CI 0.03-0.2, $p < 0.01$). Travelling outside the camp within 3 months was also associated with increasing *P. falciparum* seroprevalence (OR=1.9, 95% CI 1.3-2.9, $p < 0.01$). While individuals, returned from trip in less than 2 weeks were also found more likely to be *P. falciparum* seropositive (OR=1.9, 95% CI 1.1-3.1, $p < 0.01$).

Fever within two weeks before survey (OR=2.0, 95% CI 1.1-3.8, $p < 0.02$) and individuals who sought malaria treatment after having malaria infection (OR=2.0, 95% CI 1.0-4.2, $p < 0.05$), and individuals who taken antimalarial drugs within last two weeks before survey (OR=2.2, 95% CI 1.1-4.3, $p < 0.03$) were significantly associated with increased *P. falciparum* seroprevalence.

Risk factors related with household

Seroprevalence to *P. falciparum* was lower in the residents of households constructed of bricks or stone compared to mud (OR= 0.4, 95% CI 0.2-1.0, p<0.05). Risk of being seropositive to *P. falciparum* combined antigens was high in individuals sleeping in a room with thatched roof made of grasses and palm leaves (OR= 1.7, 95% CI 0.4-6.0, p<0.01) and if eaves were present in living room. SES was not a significant risk factor for seropositivity to falciparum malaria.

Factors related with control vector

The only significant risk factor for *P. falciparum* seropositivity (related with vector control) was use of repellents/mosquitoes coils or spray cans in the sleeping room, which was associated with decreasing likelihood of *P. falciparum* seroprevalence (OR =0.6, 95% CI 0.5-0.8, p<0.01). IRS within last 12 months and use of ITN last night were not significantly associated with *P. falciparum* antibody responses.

5.2.1.3.4. Mul variate variate Analysis

In the multivariate model the following exposure variables remained significant (Table 5.7).

Individual risk factor: An increase in seropositivity to *P. falciparum* antigens was seen with increasing age (6-20 years: OR =1.8, 95% CI 1.1-3.1, p<0.03 and >20 years: OR =2.8, 95% CI 1.7-4.5, p<0.01). Staying in the camp from last 6 months in camp of resident was associated with reduction in seropositivity to *P. falciparum* (OR =0.6, 95% CI 0.5-0.8, p<0.01).

Household risk factors:

Household constructed of bricks or stones was associated with reduce risk to *P.falciparum* seropositivity (OR =0.4, 95% CI 0.2-0.9, p<0.03). Thatched roofs made of grasses and palm leaves were associated with increased risk to *P.falciparum* seroprevalence (OR =1.5, 95% CI 1.0-2.4, p<0.01). Rooms with no eaves were also associated with lower risk of being seropositive or negative to *P. falciparum* antigens (OR =0.6, 95% CI 0.4-1.0, p<0.01).

Vector control measures:

Personal protection from mosquito vectors by using repellents or coils or spray cans was significantly associated with lower risk of *P. falciparum* seropositivity (OR =0.6, 95% CI 0.5-0.8, p<0.01).

Table 5.7 Univariate and mul variate analysis for *P.falciparum* seroposi vity

<i>P.falciparum</i> Seroposi ve	OR (Univariate)	p<	OR (Mul variate)	p<
Age groups				
1-5	1		1	
6-20	1.9(1.1-3.2)	0.01	1.8(1.1-3.1)	0.02
>20	3.1(1.8-4.9)	0.01	2.8(1.7-4.5)	0.01
Stay last 6 months				
No	1(base)			
Yes	0.1(0.03-0.2)	0.01	0.2(0.1-0.5)	0.01
Outside travel within 3 months				
No	1(base)			
Yes	1.9(1.3-2.9)	0.01		
Return recent trip				
No trip	1			
<2 weeks	1.9(1.1-3.1)	0.01		
< 4 weeks	1.7(0.7-4)	0.24		
> 4 weeks	2.1(0.9-4.7)	0.06		
Fever in the previous 2 weeks				
No	1(base)			
Yes	2.0(1.1-3.8)	0.02		
An malarial drug taken within last 2 weeks				
No	1			
Yes	2.2(1.1-4.3)	0.02		
Sought malaria treatment before				
No	1(base)			
Yes	2.0(1.0-4.2)	0.05		
Household construc on				
Mud	1(base)			
Bricks/Stones	0.4(0.2-1.0)	0.05	0.4(0.2-0.9)	0.03
Roof				
Iron sheets and rods	1(base)			
Thatched grass	1.5(1.0-2.4)	0.03	1.8(1.2-2.9)	0.01
Eaves				
Open	1(base)			
Closed	0.6(0.4-0.9)	0.02	0.6(0.4-1.0)	0.05
Windows glazed				
No	1(base)			
Yes	0.7(0.6-0.9)	0.03		
Self mosquitoes protec on (coil/repellents/spray can)				
No	1(base)			
Yes	0.6(0.4-0.8)	0.01	0.6(0.4-1.0)	0.03

5.2.1.4. *P. vivax* vs. *P. falciparum* seroprevalence and its risk factors

In the univariate logistic regression model exposure variables, which were statistically significant for both *P. falciparum* and *P. vivax* were included: Age group, outside travel within 3 months, return from recent trip (<2week, <4weeks or >4weeks), fever within two weeks, antimalarial drug taken within last two weeks prior to survey, sought malaria treatment, presence of eaves in sleeping room, presence of glazed windows and personal protection from mosquitoes vector (coils/repellents/spraycans). Residing for the last 6 months in the camp, households built of bricks or stones and iron sheets roof were significantly associated with lower seroprevalence only to *P. falciparum* (see Table 5.7). While sleeping with animals in the same courtyard and being male were significantly associated with increased seroprevalence to *P. vivax* (Tables 5.6).

In final multivariate logistic regression model, the risk of being seropositive to both *P. falciparum* and *P. vivax* was increased with increasing age and decreased by using mosquitoes coils/ repellents or can spray. However, fever within two weeks prior to survey and sleeping with animals in the same courtyard were significantly associated with increased seroprevalence to *P. vivax* but were not statistically significant to *P. falciparum* seroprevalence (Table 5.8). Conversely, the risk factors which were significant only in the multivariate logistic regression model of *P. falciparum* included stay last 6 months in the camp of residence, household construction, roof and presence of eaves in the sleeping room (Table 5.7).

5.3. Discussion

Results of this chapter are from the population based household malaria survey in five Afghan refugee camps of KP, Pakistan which aimed to provide information on the potential risk factors involved in exposure and infection of malaria in the study area. It is expected that these results will be prove useful in assessing potential malaria risk factors and improve the malaria control programs targeting of interventions.

Logistic regression model was used for the statistical analysis. In this analysis several factors relating to demography (age, gender), recent medical history (fever), travel and travel history, main material of the room's wall, roof and floor, IRS in the past 12 months, ownership and use of ITN, use of repellents, animal ownership and SES of household were used as a potential risk factors of infection and exposure to both *P. vivax* and *P. falciparum* in the study area. Age is well known confounder, was adjusted for in all multivariate logistic regression analysis.

5.3.1. Individual risk factor

Data from this study show that all age groups were exposed to *P. vivax* infection, but the OR of *P. vivax* infection when detected by RDT was reduced in age group >20 OR= 0.4(0.2-0.7), $p < 0.01$ as compared to children of age of five or less than five years, which was consistent with the situation of malaria (mostly *P. vivax*) in low endemic areas (Ramachandran, 2010). The prevalence of *P. vivax* infection detected by RDT amongst children (1-5) was 39.6%, in young age group (5-20) was 43.8% and in individuals with age more than 20 years was 16.6%. A previous study in Ethiopia suggested that prevalence of malaria infection (mostly *P. falciparum*) was highest in children and females (Ayele et al., 2012). Another study in Ethiopia also revealed that a unit increase in age resulted in 3% reduction in odds of a positive malaria test (Ayele et al., 2012). Where in lowland Africa proportion of positive malaria infection (mostly *P. falciparum*) decreased with increasing age, as immunity thought to build up readily due to repeated exposure during childhood (Ramachandran, 2010).

Serological data for both *P. vivax* and *P. falciparum* showed age-dependency in antibody responses; an expected result of the acquisition of antibody response with increasing age due to repeated exposure (Corran et al., 2007, Bousema et al., 2010c, Cook, 2010), which suggests a level of consistency in exposure in the study area.

Staying in the camp of resident from the last six prior to survey was associated with lower risk of both *P. vivax* (RDT/PCR) and *P. falciparum* (antibody responses). Travelling history was used to investigate whether movement of local resident to other endemic areas was a risk factor of malaria in the camps. Previous studies, in Mexico (Danis-Lozano et al., 2007), Thailand (Sornmani et al., 1983, Fungladda et al., 1987) and India (Ramachandran, 2010) also showed the association of malaria and traveling history (Danis-Lozano et al., 2007, Moore et al., 2004, Osorio et al., 2004). Rogelio et al suggested the movement of local resident to other neighbouring endemic areas is an important risk factor of malaria in low transmission settings (Danis-Lozano et al., 2007). These findings of the above mentioned studies from similar low endemic areas are similar with results from this study. Similarly, a case-control study conducted in Colombia, showed that travelling to an endemic area 8–14 days before disease onset was the strongest risk factor for both *P. falciparum* and *P. vivax* malaria (Osorio et al., 2004).

Survey data showed that risk of being infected with *P. vivax* (RDT/PCR) was higher in the people who had fever within 24 hours or two weeks before the survey was conducted as compared to one who had no fever. Individuals with fever were expected more likely to be parasite positive. In case of exposure to malaria (antibody responses) as an outcome, only fever within 2 weeks prior to survey was associated with *P. vivax* seroprevalence, as antibody responses represents past exposure to malaria. Similarly individuals who were treated against malaria before were also significantly associated with *P. vivax* infection detected by both RDT and PCR.

5.3.2. Household risk factors

In the final multivariate logistic regression household construction (mud), eaves in sleeping room and thatched roof made of grasses and palm leaved were significantly associated with increased exposure to *P. falciparum* exposure (antibody responses), for details see Table 5.7.

Houses built of mud with thatched roof, eaves in the sleeping room, and non glazed windows are commonly found to be at significant higher risk for malaria (Ayele et al., 2012). Because iron roof, houses constructed of bricks are thought to reduce contact with the mosquitoes via a reduction in the number of entry points to the house hence reduces infection when compared with poorer house structure (Ayele et al., 2012). Gamage-Mendis *et al.* showed that there was significantly ($p < 0.01$) higher malaria incidence rate (21.2%) in the poorly constructed houses (incomplete, mud, or palm walls, and palm thatched roofs) than better constructed houses (bricks/stones) (10.5%) (Gamage-Mendis et al., 1991).

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 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).

Low SES appears was a risk factor for the prevalence of *P. vivax* infection (PCR). Other studies also had shown lower socio-economic factors related to high malaria risk (Thang et al., 2008, Cook, 2010, Feachem et al., 2010). SES was also found significant risk factor of malaria prevalence in a study conducted in Ethiopia (Ayele et al., 2012). This may be due to a variety of factors such as being less able to afford antimalarial control measures (IRS, ITN, repellents, fan and poorly built houses as mentioned above) to reduce the risk of transmission. Therefore, the risk of malaria might be higher for households in a lower socio-economic bracket than higher status, which

suggests that the poor are less likely to use these preventative measures mentioned above to effectively reduce the spread of malaria. Possible explanation for the increased risk of malaria seen in individuals living in a poorly built house and with low SES may be a combination effect of both of these risk factors which means may be both risk factors are collinear.

Increased *P. vivax* sero-prevalence was associated with sleeping of study participants with animals in the same courtyard (OR= 1.6, $p<0.01$). However, this association was not significant risk factor for *P. vivax* infection and *P. falciparum* seroprevalence. A study carried out in Afghan refugee school children, to examine that cattle might protect occupants against malaria through zoonophylaxis, found a significantly greater parasite prevalence (15.2%) in children of families that kept domestic cattle in house courtyards was observed than among those which did not (9.5%) (Bouma and Rowland, 1995). Previous studies conducted in Ethiopia had also shown that sharing of house with livestock increased the risk of fever (Deressa et al., 2007, Graves et al., 2009). Domestic animals may compound the risk rather than decrease transmission of malaria were suggested to support the prediction of Sota-Mogi theoretical model (1989), especially, when vectors are zoophilic, rate of infection is low, and a high human:cattle ratio (Mathys, 2010, Bouma and Rowland, 1995).

5.3.3. Vector control measures

This cross sectional study collected information only about coverage of Insecticide Residual Spray (number of houses with IRS within 12 months) and did not deal with the efficacy of IRS. The percentage of houses that reported having had IRS within 12 months in all the camps data was 5.4%, ranged between 2.4-10.5 % with lowest percentage in Baghicha (camp 2) and highest in Zangal patai (camp 5). According to this study, IRS within 12 months was not significantly associated with low prevalence of infection or exposure to both *P. falciparum* and *P. vivax*. Possible reasons for the non significant association between IRS within 12 months and malaria infection and exposure may be that the spraying was not conducted by any governmental organization or NGO but by a household head who were not trained. As such they may be unaware of the careful choice of insecticide as certain vector species are resistant to DDT and organophosphate insecticide (Rowland and Nosten, 2001) or the proper time of IRS which might exerts maximum effect before transmission season (Rowland and Nosten, 2001). It may also be that at such low coverage no effect was likely to be observed.

However, previous studies to determine the efficacy of IRS showed 50%-60% protective efficacy against *P.falciparum* and 40%-50% against *P.vivax*, by using malathion and lambda cyhalthrin in the Afghan refugee camps (Rowland et al., 1994). Another study conducted in nomadic Afghan refugees showed that spraying tents with permethrin lowered the risk of *P.falciparum* malaria by 66% (Bouma et al., 1996). In a comparative study of ITN and IRS in refugee camps in Pakistan and Tanzania, both IRS and ITN were observed to be effective (Curtis et al., 1999, Rowland, 1999a).

The study also collected information about the number of household-owned ITN and its use on the night before the survey to determine association of both exposure variable (ITN ownership and ITN use night prior to survey) with prevalence of infection and exposure to malaria. According to results the overall ITN coverage was 48.6%, ranged from 78.7% in camp 2 to in 20% camp 5. This study showed that sleeping under ITN on previous night before the survey was associated with lower risk of vivax malaria infection (both RDT and PCR). The single night before the survey is unlikely to have not accurately captured the net use over a longer period, but can reflect the behaviour of people towards ITN use. As these results only deal with the ITN coverage in the camps

and its use, they therefore cannot be directly compared with previous studies about ITN efficacy in these camps that were based on different design. Secondly, these studies were also done at the time when parasite rate may be much higher (as was reported previously) as compared to the present study when the parasite rate is much reduced (BHU data). For example, a previous household randomized trial of ITN in Afghan refugee camps in KP, Pakistan had shown 61% protection against *P.falciparum* and 45% protection against *P. vivax* (Rowland et al., 1996, Rowland et al., 1997b). Another household randomized trial in Afghan refugee camps where, top sheets, blankets and Islamic head-body veils were treated with permethrine, which gave 64% protection against *P. falciparum* and 38% against *P.vivax*, and these were suggested as a good means for short term epidemic control of malaria (Rowland, 1999b).

A study in Thailand to evaluate the effect of irregular and non use of mosquito nets, revealed an increased risk of malaria (2.5 fold) in nonusers, in irregular users (1.5-6.4 fold) than regular users. According to a review on epidemiology and control in refugee camps and complex emergencies, there is very little or no tradition of using net for protection against mosquitoes in south and central Asian countries (Rowland and Nosten, 2001). Similarly a study in Ethiopia also found low risk of malaria for mosquito nets users compared to non users (Ayele et al., 2012).

The use of mosquitoes repellents/coils or can spray as a personal protective measures were also associated with a reduced risk of vivax malaria infection (RDT) and exposure to both *P. vivax* and *P. falciparum*. Similarly on several traveller sites (including Afghanistan and Pakistan) the most commonly malaria prevention measures suggested based on the risk assessment included: drugs used in the prophylaxis of malaria, bed net, insect repellent, flying insect spray, mosquitoes coils, long-sleeved clothing and specific medicines to prevent malaria (CDC, 2012b). However a systematic review of trials to determine the role of mosquitoes coils in prevention of malaria found no evidence that burning mosquito coils may led to prevention of malaria infection, but may reduce the chances of being bitten by mosquitoes (Lawrance and Croft, 2004).

All the three measures RDT/PCR and antibody responses against Plasmodium species are different. For example, RDT is commonly used to detect recent infection; PCR is used for detection of recent and submicroscopic infections; while antibody responses

are used to determine exposure to malaria parasite. But all of them are useful to measure malaria transmission and to determine its potential risk factors in an area, especially at low levels of transmission, where microscopy and RDT may not be sensitive measures of infection prevalence. This study site is a low malaria transmission intensity area, where *P. falciparum* infection prevalence was almost zero and *P. vivax* infection prevalence was very low when detected by RDT. Therefore all the three measures together may be useful to represent any likely source of exposure and infection to identify potential risk factors of malaria in an area. Similarly, in low endemic areas of Somaliland antibody responses were used to detect low levels of malaria transmission where microscopy detected no malarial infection but antibody responses against both *P. falciparum* and *P. vivax* were detected to evaluate ongoing malaria transmission (Bousema et al., 2010c). The application of these results to advise malaria control programmes of the study area to help in the control of malaria are discussed in chapter 6. In which heterogeneity in infection and exposure to malaria disease was assessed within the camps and to discuss the risk factors as being associated to living in a hot spot of malaria transmission.

Chapter Six

Identifying hot spots of malaria exposure and infection within the refugee camps

Chapter 6. Identifying hot spots of malaria exposure and infection within the refugee camps

In this chapter, heterogeneity in infection and exposure to malaria disease was determined in the residents of five Afghan refugee camps in KP, Pakistan. To achieve this objective, household level GIS data was combined with infection data and serological responses to map potential foci of infection. Geographic information systems (GIS) data integrated with malaria data may provide a quick display/picture of malaria transmission to identify areas of intense or lower than average malaria exposure. To analyse and visualize clusters of malaria transmission, GIS programmes, SaTScan and ArcGIS were used.

Malaria mapping has become increasingly common throughout the world over the past decade to provide a spatial overview of the disease which permits spatial patterns of the disease. Aid agencies who are assisting malaria control and elimination both financially and technically in malaria-prone countries focusing on the mapping of malaria to provide specific spatial information of the disease (Map, 2013, EMRO, 2013, WHO, 2011a). Mapping of malaria is done on variety of scales but mostly on large/global or country level and the utility of spatial analysis on small admin zone is beginning to be used (Bousema et al., 2012).

The distribution of malaria risk and its burden is unequal within the population (Greenwood, 1989, Woolhouse et al., 1997, Carter et al., 2000). The factors which are responsible for the heterogeneity in malaria is not fully described or explored yet the most commonly observed factors include, environmental and behavioural factors and use of interventions (EMRO, 2001). Although there is spatial heterogeneity in the risk of infection, but there is also similar heterogeneity for infectiousness. Because a small number of infected individuals can most likely increase transmission by infecting a huge number of mosquitoes (Smith et al., 2007), which can increase basic reproductive number of malaria parasites (R_0) 2-4 fold (Woolhouse et al., 1997).

Therefore, it would be potentially more useful and efficient to control and target malaria to the areas that contribute disproportionately to malaria transmission and focus efforts on a relatively small proportion of people (Smith et al., 2007). The

usefulness of a targeted approach is thought to be more beneficial in areas where transmission has decreased but reservoirs of infection exist as hot spots of malaria transmission (Bousema et al., 2010a, Bousema et al., 2012). Specially targeting hot spot of infection in the malaria endemic areas will most likely reduce transmission and also save money and time by targeting control to these areas (Fegan et al., 2007, Bhattarai et al., 2007). So, controlling malaria at individual level may not only direct benefits for the individuals who are included in the control effort but also at the community level by interrupting onward transmission.

Variation in the risk of malaria within populations can be used for targeted interventions to identify hot spots of malaria transmission (Bousema et al., 2010a). However, a prerequisite for this is accurate information on the geographical distribution of malaria transmission intensities (Gustavo Brêtas, 1996, EMRO, 2001, Carter et al., 2000). Mosquito abundance (Bousema et al., 2010a), elevations in malaria incidence (Clark et al., 2008, Bousema et al., 2010a, Bejon et al., 2010, Ernst et al., 2006), asymptomatic parasite carriage (Ernst et al., 2006, Bejon et al., 2010) and serological responses to malaria specific antigens (Bousema et al., 2010a, Bousema et al., 2010c) have been used previously to describe spatial patterns in malaria transmission.

The most direct evidence of hotspots of malaria transmission can be described by increased exposure to infectious mosquito bites. But EIR has low sensitivity at low transmission intensity and will require huge sampling which is laborious and time consuming process which make entomological detection of hotspots logistically unattractive (Bousema et al., 2012). Asexual parasite carriage was observed to be a more stable indicator of hotspots of malaria transmission (Bejon et al., 2010).

Similarly, malaria-specific antibody responses were suggested to be the most robust indicators of hotspots of malaria transmission and were previously used to show small scale variations in malaria exposure (Bousema et al., 2010a, Bousema et al., 2010c, Wilson et al., 2007). Because antibody responses are long-lived, accumulate with age due to cumulative exposure and hotspots of malaria transmission were observed to be unaltered after overall malaria transmission is reduced (Ernst et al., 2006, Bautista et al., 2006, Gaudart et al., 2006). Additionally, antibody prevalence was higher than both

measures of parasite prevalence (RDT/PCR) and this extra sensitivity may provide more detailed examination of the spatial patterns in the study area. RDT, PCR and serology were employed together to detect any likely source of ongoing transmission in this low transmission intensity area. Although, all the three measures have their own biases, each contributes essential information about transmission dynamics especially in low malaria endemic areas.

6.1. Analysis

Spatial patterns in malaria transmission at a household level were determined using parasite prevalence rates measured by RDT, PCR and seroprevalence. To determine the infection status CareStart 3-line Pf (HRP2) Pan (pLDH) combo RDT was done on all samples (2526) with observed 98.6% sensitivity previously (Mikhail et al., 2011). Spatial analysis to detect hot spots of parasite prevalence (areas whereby infection and disease are concentrated in a small proportion of individuals) measured by RDT was done for all camps. Spatial analysis to detect hot spots of parasite prevalence measured by nested PCR was done for camp 3 only. This was because a subset of samples (discussed in chapter 2 in detail) was assayed by PCR in all the four camps except for camp 3 from which all samples were assayed. Antibody responses were combined to both antigens against *P. vivax* (PvAMA+PvMSP) and *P. falciparum* (PfAMA+PfMSP) antigens to enhance the power for mapping seroprevalence data.

Before detection spatial clusters of seroprevalence antibody responses to *P. vivax* and *P. falciparum* were log transformed and adjusted for age as described previously (Wilson et al., 2007, Bousema et al., 2010c, Cook, 2010). This was done to see whether antibody responses were higher or lower than expected for any given age. For each individual and each antigen, age dependency of antibody responses was determined by visual assessment against a Lowess lines on age-normalized OD. The age dependency of antibody response was confirmed by using linear regression on different age blocks to see if the slope or significance level changed. If the association was non-significant then did not fit a line but in case of statistically significant association, a linear line was fitted and deviation from this line was determined.

Spatial analysis software provides greater opportunity to understand disease distributions, risk factors, and changes to population health over time and space. To assess spatial heterogeneity *i.e.* the presence of hot/cold spots in *P. vivax* and *P. falciparum* infection/seroprevalence within camp SaTScan software version 9.1.1 was used. SaTScan is the most commonly used software package for detection of disease cluster in a population. However, SaTScan only implements scan statistic methods and do not deal with modeling-based approaches (Robertson and Nelson, 2010).

The most significant clusters (potential areas with significantly higher or lower numbers of exposure than the mean with P value ≤ 0.05) were detected by using 999 Monte Carlo replications to ensure sufficient power to define clusters. The standard purely spatial scan statistic used a circular window on the map but an elliptic shaped window can also be used, which provides slightly higher power for true clusters than circular. In this study for systematic scanning of each studied camp, a circular-shaped window was used instead of elliptical because the coordinates collected by GPS were in decimal degrees units, while elliptical windows uses coordinates in Cartesian units only.

Maximum spatial cluster size was selected as 50 percent of the population at risk. The circular windows were restricted to 1km radius and upper limit was specified as 50% of the village population. For each cluster an expected number of cases were calculated and was compared to the observed number of cases. To determine high seroprevalence (0/1) and parasite prevalence (0/1) clusters a Bernoulli model was used, which is used for cases and non-cases (people with or without a disease) represented by a 0/1 variable. While for scanning high antibody responses (age adjusted normalized ODs, continues values) clusters, a normal model was used to fit the continuous nature data. The same constrains as described above were used for this model.

Integrated maps showing basic features and predicted hotspots were produced in ArcGIS version 10 (Environmental Systems Research Institute). Visual display of individual level attributes was done using the GPS coordinates for each household and displayed using ArcGIS 10 (ESRI Inc. USA). Main roads and inland water bodies (canals, streams and rivers) longitude and latitudes points were assembled, developed and computed using Google earth.

6.2. Results

Total number of *P. vivax* infections detected by RDT was 96/2526, the number ranged between 2-49 with lowest in camp 1 and highest in camp 3. No RDT positive individual was observed for *P. falciparum* in four camps except for a single positive individual in camp 5. Therefore spatial analysis of *P. vivax* infection (RDT) was conducted in all camps but not for *P. falciparum* infection due to zero prevalence. PCR was done on all samples only for camp 3 which detected 79/505 positive individuals. In other four camps (camp 1,2,4 and 5) 120 plus all RDT positive samples were assayed by PCR for each of the camps (discussed in detail in chapter 4). Hence, to observe hot or cold spots of *P. vivax* infection detected by PCR was done only for camp 3. Again due to limited number of *P. falciparum* infections (7) observed by PCR in camp3, spatial analysis to see significant cluster of infection in the camps was not possible. However, spatial analysis was carried out for all the camps and for both *P. falciparum* and *P. vivax* using seroprevalence data.

Parasite prevalence and seroprevalence in individuals less than 5 years of age were used to represent local and recent transmission. Children in age group 1-5 are most likely to represent recent exposure and are less likely to have travelled to other high transmission area. Antibody response (log transformed normalized ODs) to both *P. falciparum* and *P. vivax* (AMA-1 and MSP-1₁₉) were adjusted for age to represent whether antibody responses were higher or lower than expected for any age given age group. Heterogeneity in prevalence of malaria infection and exposure was also assessed considering all ages in the study because certain factors will only be observed in adults (e.g, past malaria exposure, movement to other endemic areas) and to provide a more comprehensive assessment of transmission.

6.2.1. Spatial Patterns in infection and seroprevalence in Camp1

6.2.1.1. Children under or equal to five years of age

No significant cluster of infection and seroprevalence were observed in camp 1 this is likely due to the fact that as infection detected by both RDT and seroprevalence were very low among individuals equal to or less than five years age.

6.2.1.2. Clusters of age adjusted antibody responses

No significant cluster of age adjusted antibody responses against *P. vivax* were detected in camp1. While one significant hot spot of age adjusted *P. falciparum* OD was observed in this camp (Radius 0.24 km, Number of households in the hot spot=63, $p<0.01$) (Figure 6.2.A).

6.2.1.3. Seroprevalence and infection cluster in individuals of all ages

In camp1 the SaTScan analysis carried out for detection of high or low *P. vivax* antibody prevalence clusters considering individuals from all ages, showed one hot (Hot spot: Radius 0.12 km, Number of cases=31, Expected cases=13.17, $p<0.01$) and one cold spot (Radius 0.22 km, Number of cases=16, Expected cases=36.53, $p<0.01$) (Figure 6.2B).



Figure 6.1Aerial view of camp 1

This bird's eye view of the camp was captured from Google earth with eye altitude 1.25 Km. The figure is showing the households position relative to their distance from water bodies, roads and BHU. The small pink dots represent selected households for the study.

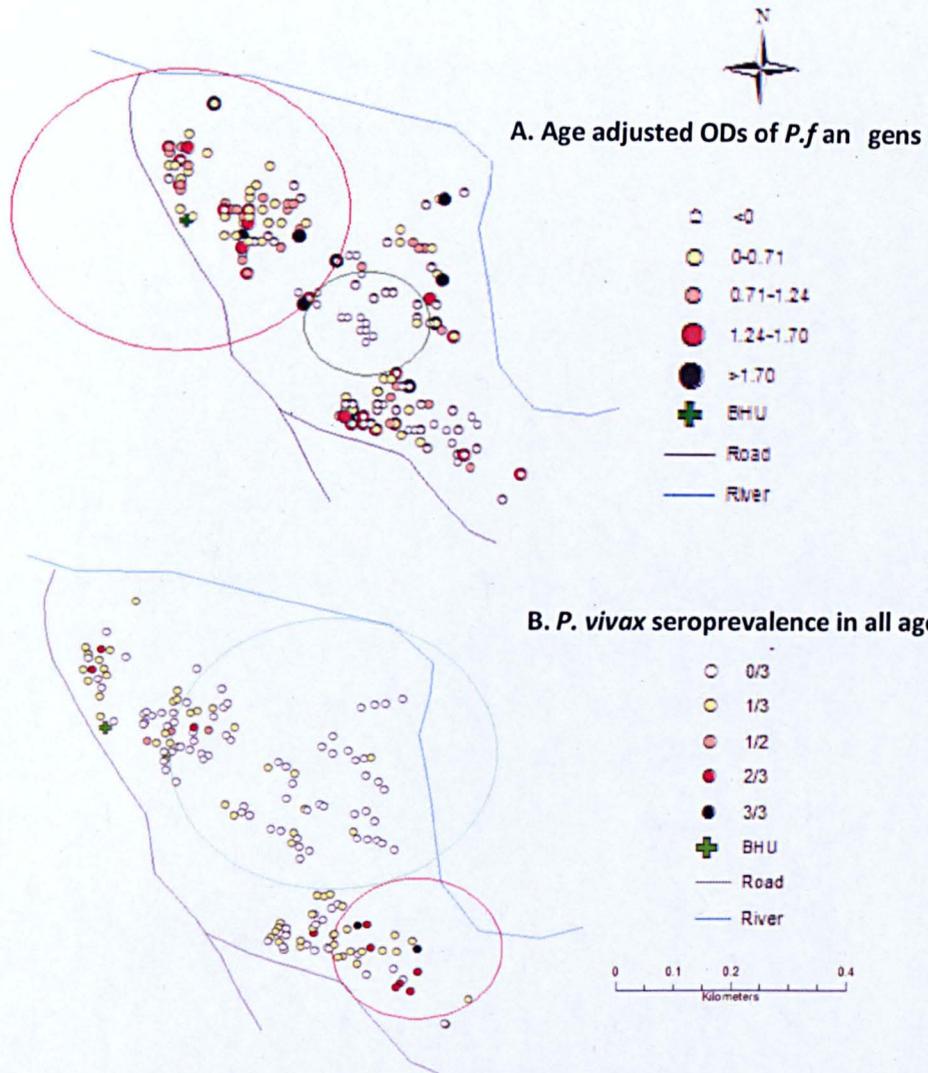


Figure 6.2(A) Hot and cold spot of age adjusted operational density values against *P. falciparum* and (B) seroprevalence in all ages against *P. vivax*

In figure A colored dots indicate mean age-adjusted operational densities per household for combined seroreactivity to *P. falciparum* (MSP-1₁₉ and AMA-1). The red circular window indicates a statistically significant cluster of higher *P. falciparum* and *P. vivax* seroreactivity, while the green circular window represents cold spot. In figure B, Colored dots indicate mean seroprevalence in each household for combined antigens of *P. vivax* (PvMSP+PvAMA).

6.2.2. Spatial Patterns in infection and seroprevalence in Camp 2

6.2.2.1. Children under or equal to five years of age

No significant foci of infection and seroprevalence to both *P. falciparum* and *P. vivax*, either cold or hot was found for camp 2.

6.2.2.2. Clusters of age adjusted antibody responses

In camp 2 age adjusted *P. vivax* seroreactivity was significantly increased in a cluster of 49 households with $P=0.001$ (Figure 6.4.A). One significant hot spot of age adjusted *P. falciparum* OD was observed in camp 2 (Radius 0.22 km, Number of households in the hot spot=55, $p<0.01$) (Figure 6.4.B).

6.2.2.3. Seroprevalence and infection cluster in individuals of all ages

Only one *P. falciparum* highly significant hot spot with radius 0.21 km, number of cases 44, expected cases 15.9, $p<0.01$ and one cold spot was detected with radius 0.41 km, number of cases 2, expected cases 23, $p<0.01$ in camp 2 (Figure 6.4.C). This hot spot is in similar geographical location as was detected when age adjusted ODs data was used.



Figure 6.3Aerial view of camp 2

This bird's eye view of the camp was captured from Google earth with eye altitude 1.25 Km. The figure is showing the households position relative to their distance from water bodies, roads, tobacco factory and BHU. The small pink dots represent selected households for the study.

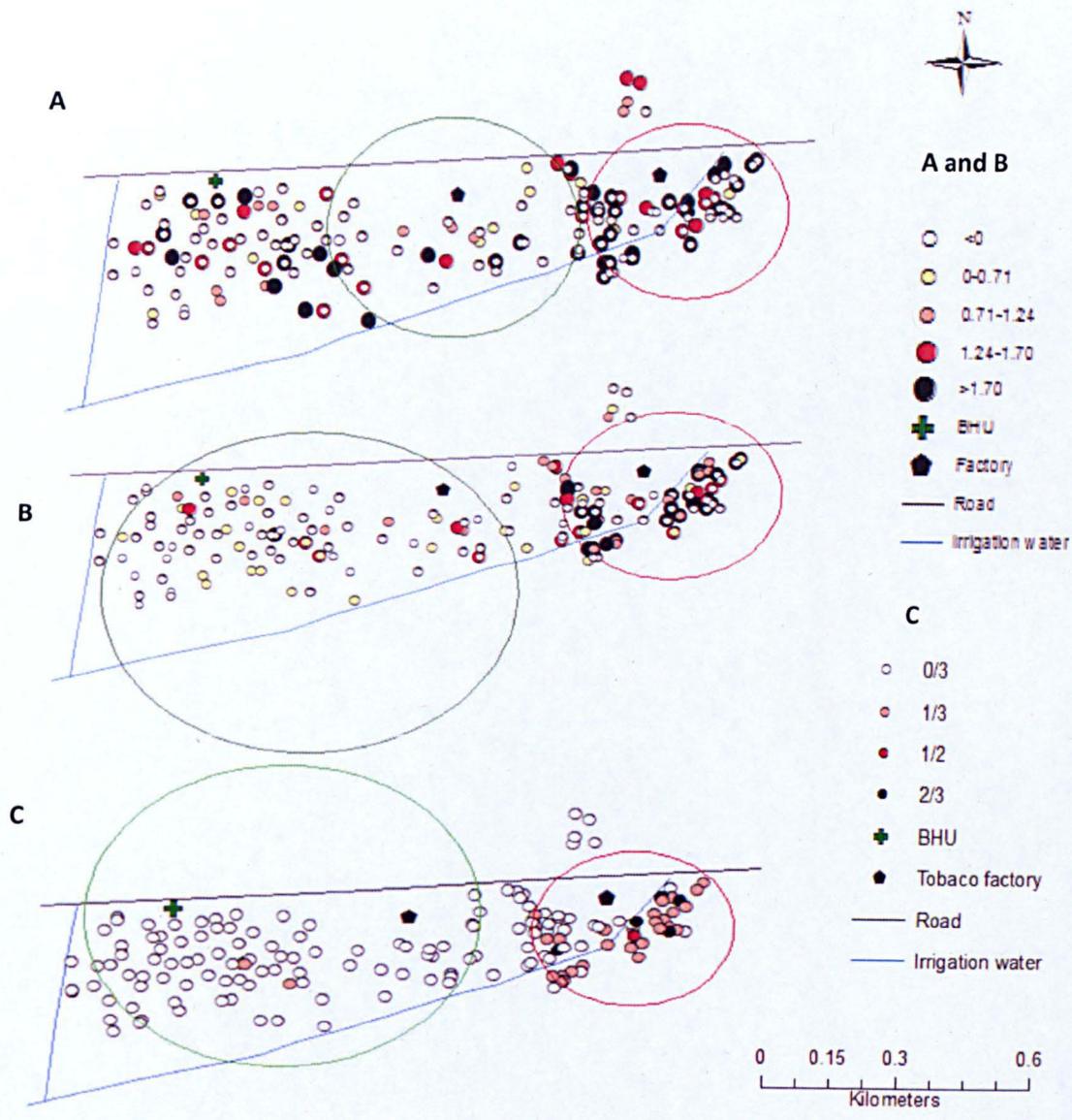


Figure 6.4(A) Spatial distribution of *P. vivax* age adjusted ODs (A) age adjusted ODs of *P. falciparum* and (C) *P. falciparum* seropositives in all ages

In figures A and B colored dots indicate age-adjusted optical densities per household for combined seroreactivity to *P. falciparum* and *P. vivax* MSP-1₁₉ and AMA-1. The red circular window indicates a statistically significant cluster of higher *P. falciparum* and *P. vivax* seroreactivity, while the green circular window represents cold spot. In figure C, Colored dots indicate mean seroprevalence in each household for combined antigen of *P. falciparum* (PfMSP+PfAMA).

6.2.3. Spatial Patterns in infection and seroprevalence in Camp 3

6.2.3.1. Children under or equal to five years of age

In camp 3 one cluster of high parasite prevalence (with 0.33 km radius, number of observed cases 19, expected cases 7.5 and $p < 0.01$ using Likelihood Ratio Test) and one of low prevalence (with radius 0.56 km, observed number of cases 0, expected number of cases 10 and with $p < 0.01$) was detected when using RDT data (Figure 6.6.A). The same hot spot of infection was also detected by using PCR data, where the number of cases observed was 16 and the number of expected cases was 6.16 (radius 0.34 km, $p < 0.01$) (Figure 6.6.B).

The analysis of cold/hot spots of sero-prevalence against *P. vivax* individual of age equal or less than five years showed only one significant hotspot in camp3. This comprised of similar households and was geographically congruous with those detected for PCR and RDT, with radius 0.41 km, 46 cases, and 26 expected cases with a $p < 0.01$ (Figure 6.6.C).

6.2.3.2. Clusters of age-adjusted antibody responses

In camp3 one cluster of higher than expected age-adjusted *P. vivax* OD values was detected, consisted of 25 households with $p < 0.01$ (Figure 6.7.A). One significant hot spot of age adjusted *P. falciparum* OD was also observed in camp 3 (Radius 0.16 km, Number of households in the hot spot=11, $p < 0.01$) (Figure 6.7.B).

6.2.3.3. Seroprevalence and infection clusters in individuals of all ages

In camp 3 one hot spot of infection prevalence (RDT) in individuals of all ages, with radius 0.33 km, number of cases 39, expected cases 16.6, $p < 0.01$ and one cold spot with radius 0.47 km, number of cases 0, expected cases 17.3 and $p < 0.01$ (Figure 6.8.A). One hot (radius 0.40 km, number of cases 2, expected cases 18.3) and one low *P. vivax* prevalence cluster $p < 0.01$ and (radius 0.56 km, number of cases 59, expected cases 37, $p < 0.01$) was detected when PCR based infection prevalence was used. This was similar in geographical position with hot and cold spots detected when RDT data was used (Figure 6.6.B). No significant hot or cold spots were found in the other four camps using parasite infection prevalence data. One hot spot (Radius 0.18 km, Number of cases=82, Expected cases=48.58, $p < 0.01$) and one cold spot (Radius 0.92 km, Number

of cases=8, Expected cases=118.59, $p<0.01$) of *P. vivax* antibody prevalence was also detected at common geographical position as hot spots detected when RDT/PCR data was used(Figure 6.6.C).

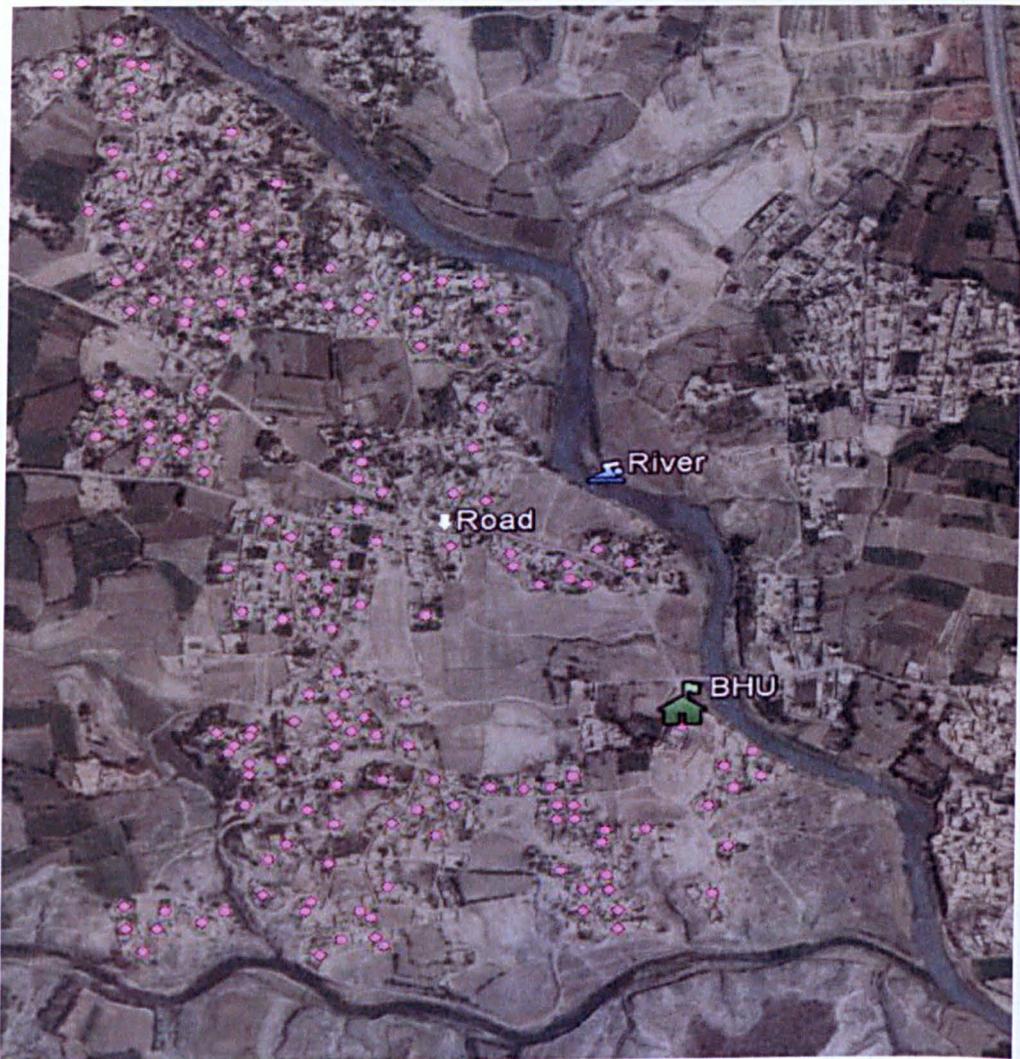


Figure 6.5Aerial view of camp 3

This bird's eye view of the camp was captured from Google earth with eye altitude 1.25 Km. The figure is showing the households position relative to their distance from water bodies, roads and BHU. The small pink dots represent selected households for the study.

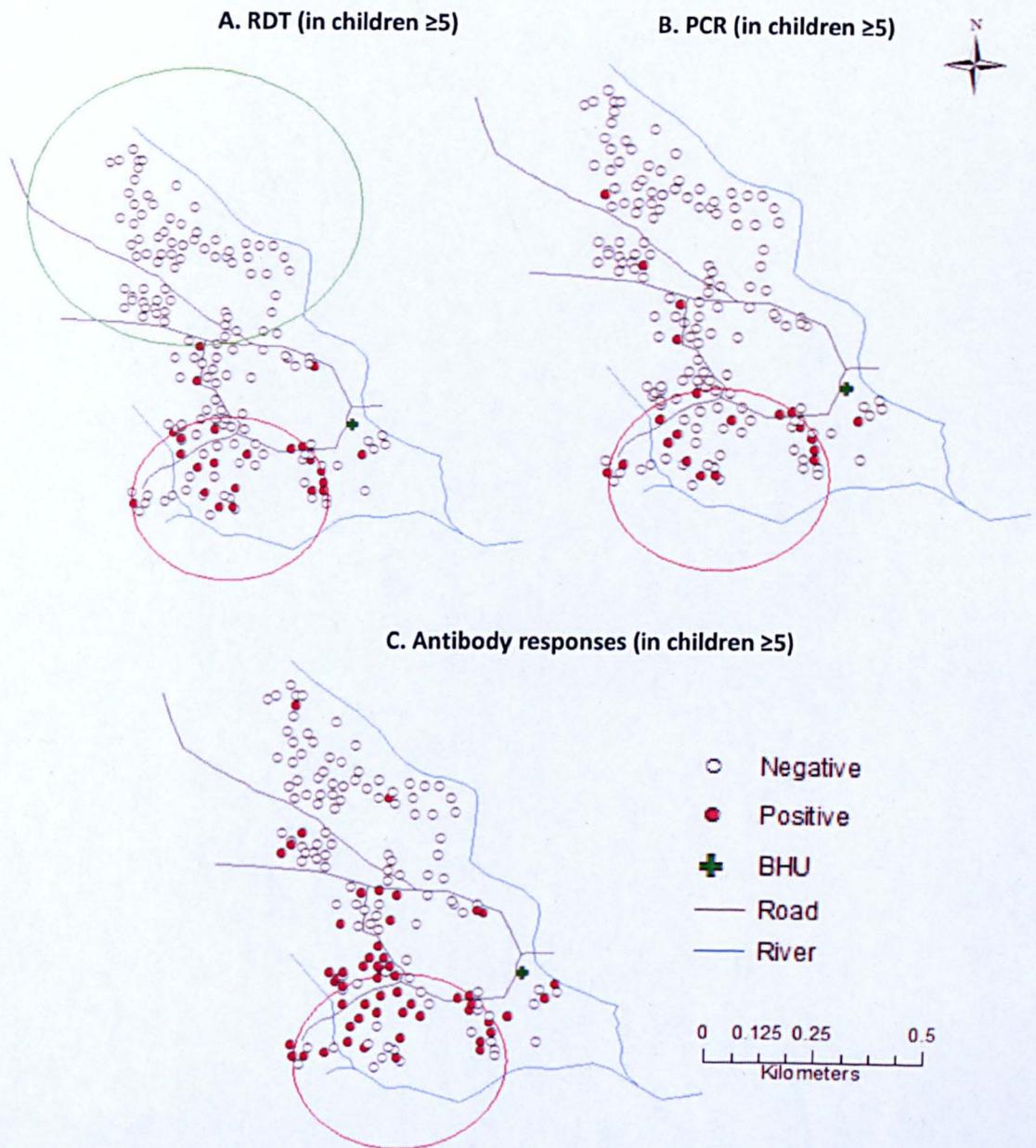


Figure 6.6 (A) Hot and cold spots of *P. vivax* infection using RDT data (B) PCR spatial distribution of parasite positive people (C) seropositive individuals against *P. vivax*, in individuals equal to or less than five years.

Household with negative infection individuals are represented by hollow circle and household with parasite positive individuals are represented by a red circle. The red circular window indicates a statistically significant cluster of higher *P. vivax* infection, while the green circular window represents cold spot.

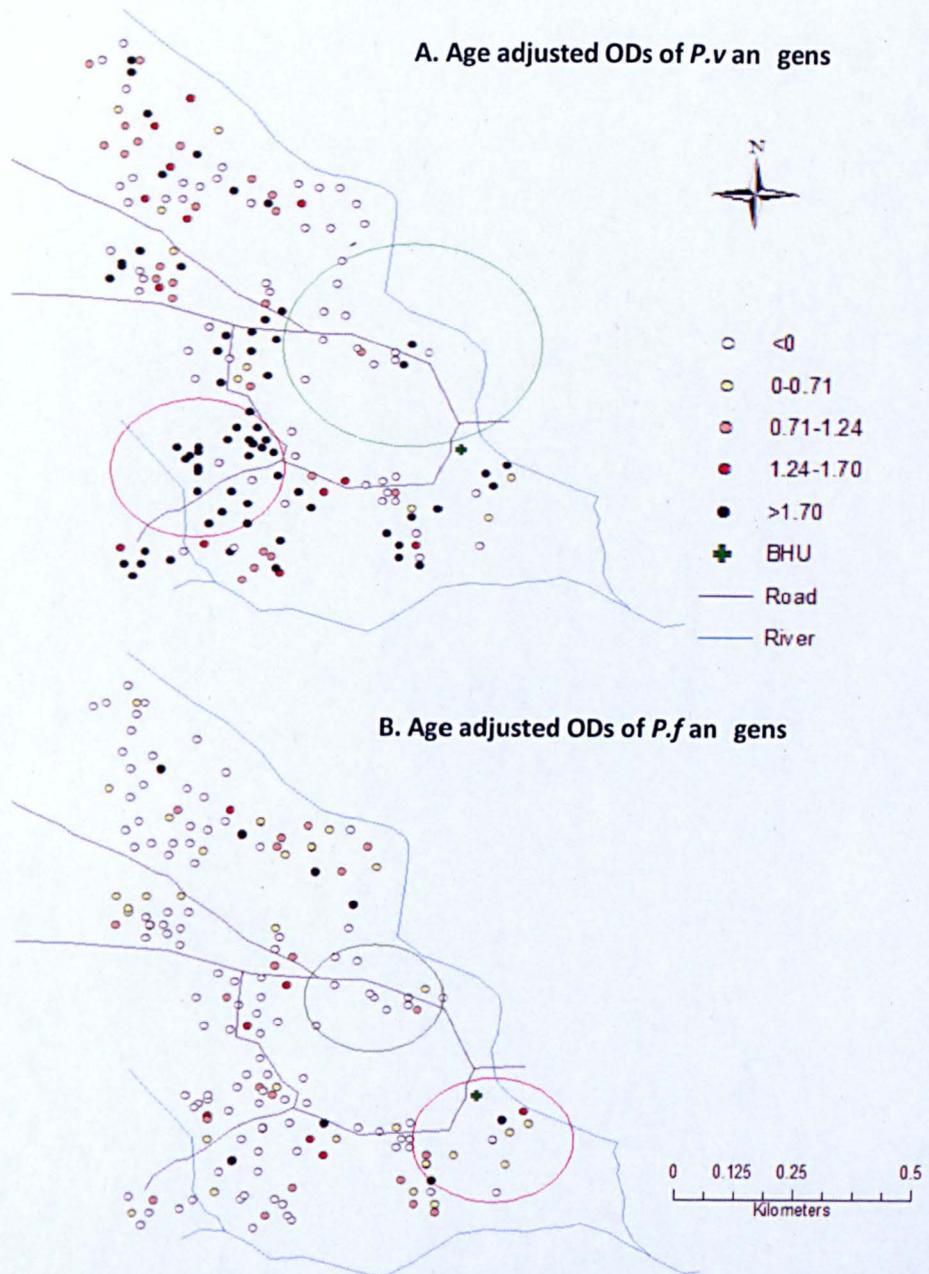


Figure 6.7 Hot and cold spot of age adjusted operational density values against (A) *P. vivax* and (B) *P. falciparum*

Colored dots indicate age-adjusted operational densities per household for combined seroreactivity to *P. falciparum* and *P. vivax* MSP-1₁₉ and AMA-1. The red circular window indicates a statistically significant cluster of higher *P. falciparum* and *P. vivax* seroreactivity, while the green circular window represents cold spot.

A. *P. vivax* infection in all ages (RDT)

B. *P. vivax* infection in all ages (PCR)

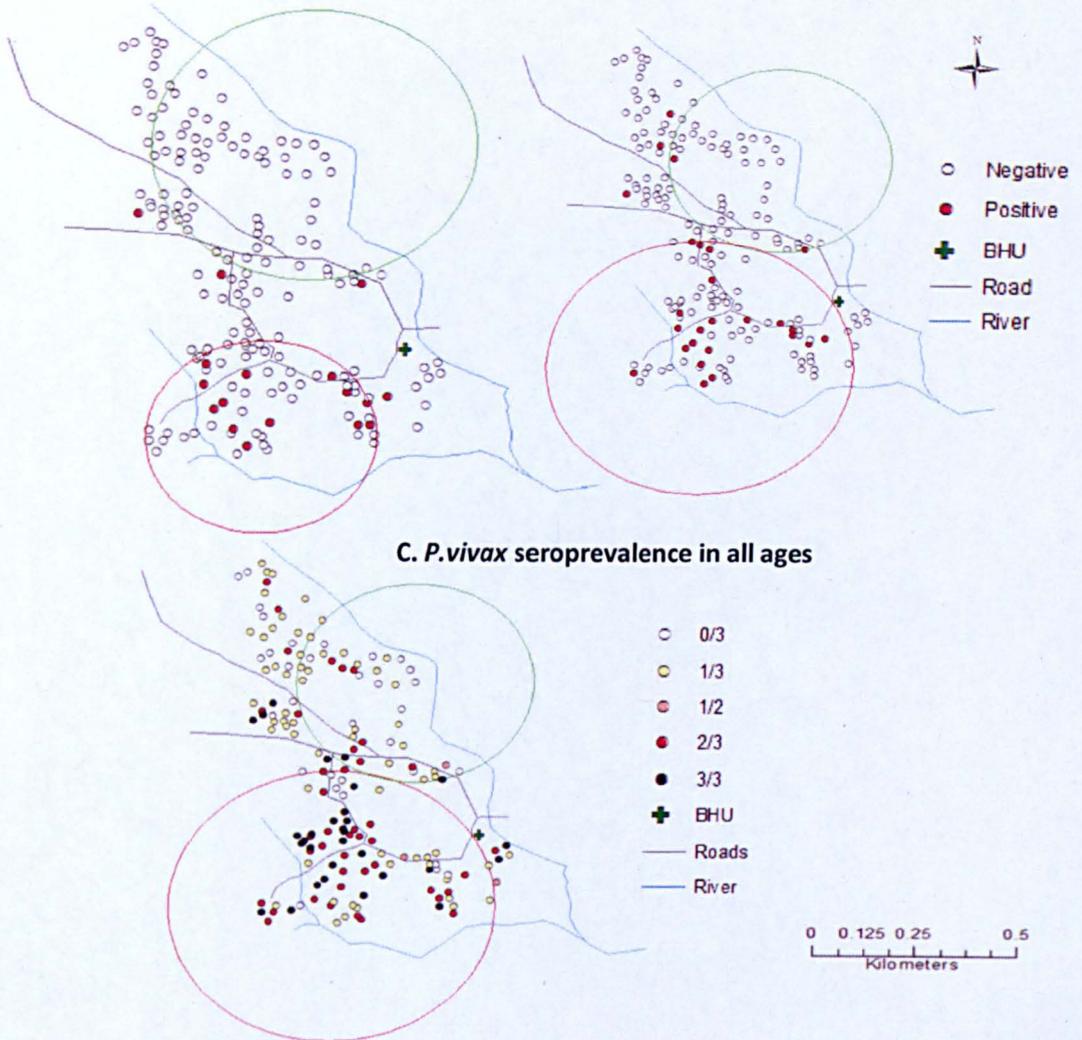


Figure 6.8 Spatial distribution of infection prevalence using (A) RDT (B) PCR and (C) seropositive individuals against *P. vivax* all ages

In figures A and B, Household with negative infection individuals are represented by hollow circle and household with parasite positive individuals are represented by a red circle. While in figure C colored dots indicate mean seroprevalence in each household for combined antigen of *P. vivax* (PvMSP+PvAMA). The red circular window indicates a statistically significant cluster of higher *P. vivax* infection and seroreactivity, while the green circular window represents cold spot.

6.2.4. Spatial Patterns in infection and seroprevalence in Camp 4

A single significant ($p < 0.01$) small hot spot of *P. vivax* age adjusted ODs with 20 households was detected in camp 4 (Figure 6.10).



Figure 6.9 Aerial view of the camp 4

This is bird's eye view of the camp was captured from Google earth with eye altitude 1.25 Km. The figure is showing the households position relative to their distance from water bodies, field, roads and BHU. The small pink dots represent selected households for the study.

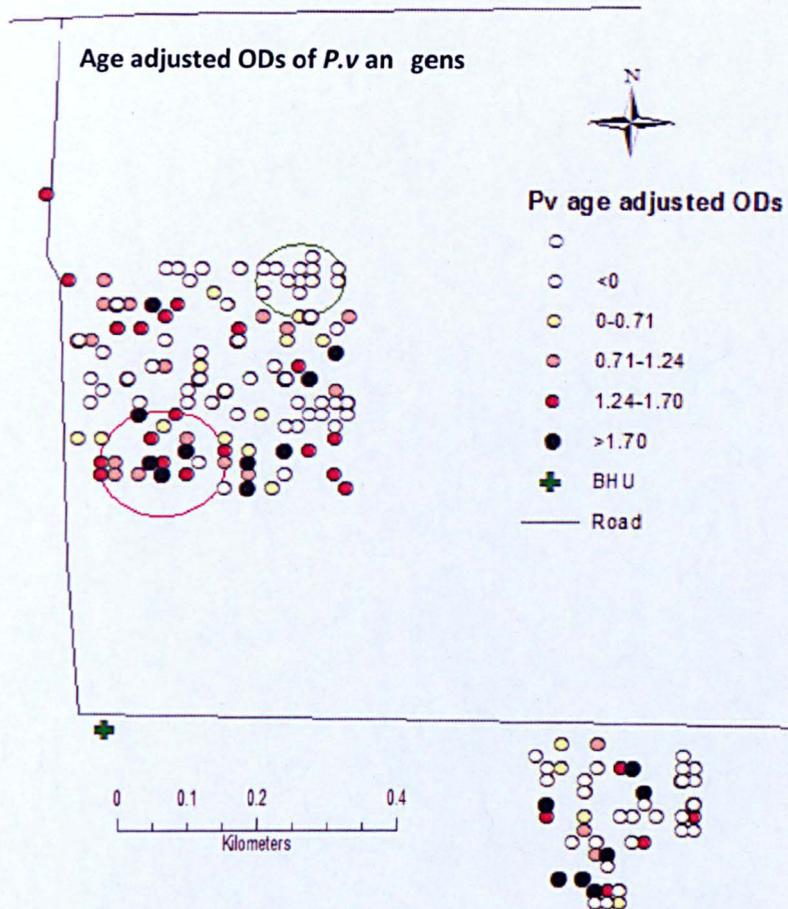


Figure 6.10 Hot spot of *P. vivax* exposure using age adjusted ODs in camp 4

Colored dots indicate age-adjusted op cal densi es per household for combined seroreac vity to *P. vivax*MSP-1₁₉ and AMA-1. The red circular window indicates a sta s cally signi cant cluster of higher *P. vivax*seroreac vity, while the green circular window represents cold spot.

6.2.5. Spatial Patterns in infection and seroprevalence in Camp 5

In camp 5 one significant hot spot of age adjusted *P. falciparum* ODs were observed (Radius 0.08 km, Number of households in the hot spot=14, $p<0.01$) (Figure 6.12.B). Two significant cold spot of age adjusted *P. vivax* OD ($p<0.01$) was also observed (Figure 6.12.A).



Figure 6.11 Aerial view of the camp 5

This is bird's eye view of the camp was captured from Google earth with eye altitude 1.25 Km. The figure is showing the households position relative to their distance from water bodies, roads, and BHU. The small pink dots represent selected households for the study.

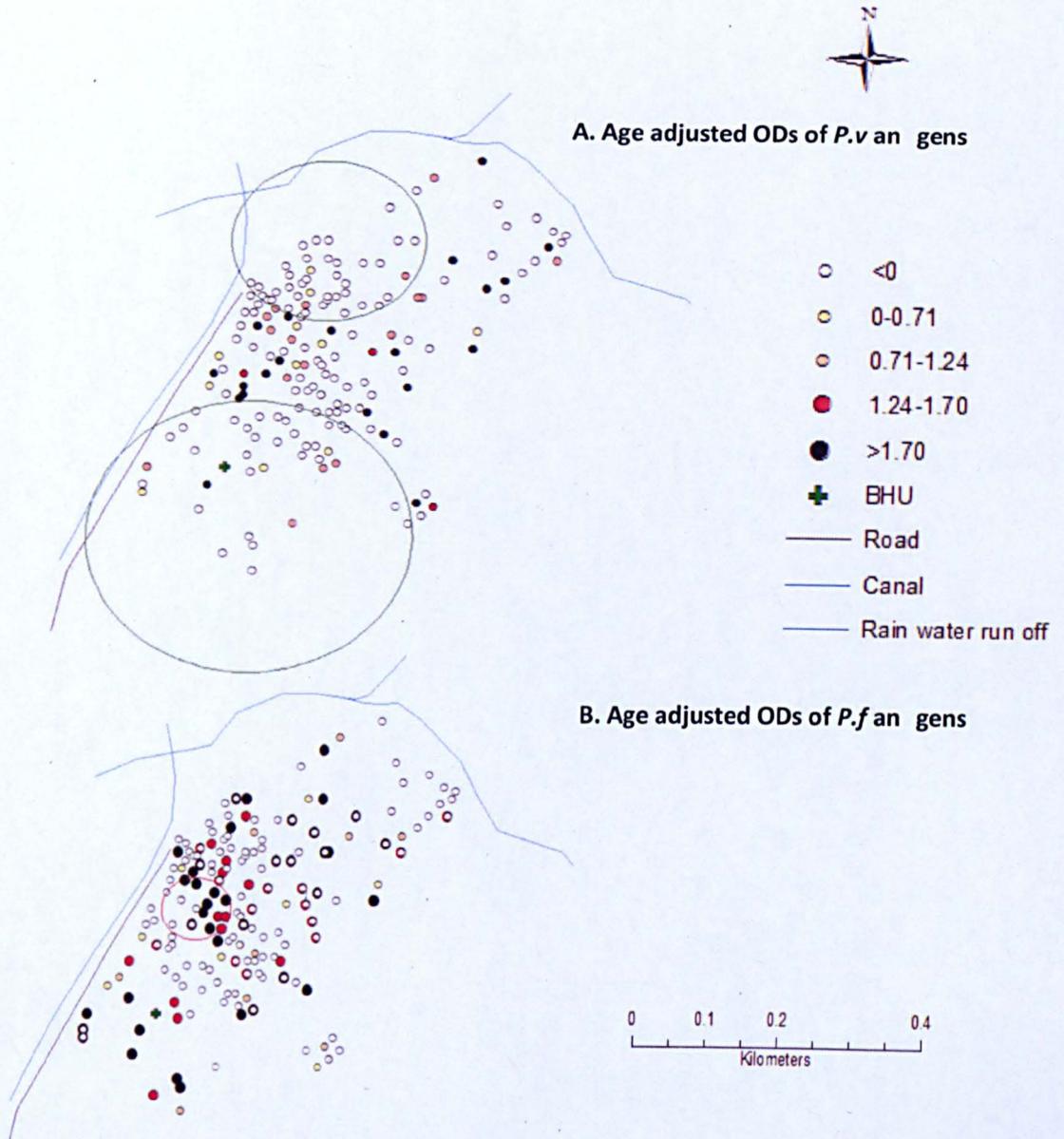


Figure 6.12(A) Cold spots of age adjusted ODs of *P. vivax* (B) hot spot of age adjusted ODs against *P. falciparum*

Colored dots indicate age-adjusted op cal densi es per household for combined seroreac vity to *P. falciparum* and *P. vivax* MSP-1₁₉ and AMA-1. The large red circular window indicates a sta s cally signi cant cluster of higher *P. falciparum*, while the green circular window represents cold spot.

6.3. Discussion

In this chapter the spatial variation in malaria infection and exposure was examined in the five Afghan refugee camps by integrating survey data described in chapter 4 and chapter 5 with household level GPS in KP, Pakistan. Spatial statistics were generated using SaTScan software to detect areas of higher and lower than expected malaria transmission. Areas that had higher or lower number of individuals who were parasite positive and having positive antibody responses than expected were defined as hot or cold spot respectively.

Evidence of heterogeneity was observed in prevalence of *P. vivax* infection and antibody responses to both *P. falciparum* and *P. vivax* within the camps. In camp 1 one cluster of high age adjusted ODs against *P. falciparum* and high seroprevalence against *P. vivax* considering individuals from all ages was detected. In camp 1 the hot spot of antibody prevalence to *P. vivax* in all age groups was detected and is probably due to the fact that these households were found very close to water logged rice fields, and far from BHU. In camp 2 one hot spot for each malaria species (*P. falciparum* and *P. vivax*) was detected by using age adjusted ODs data. For *P. falciparum* another hot spot was detected when seroprevalence data of all age groups was used in camp 2. All the three hot spots observed in camp 2 (one *P. vivax* and two of *P. falciparum*) were detected in similar geographical position. The hot spots were comprised of households that were scattered along a small irrigation water canal and near tobacco factory that may be one of the reasons for hot spot as several studies previously mentioned the proximity to water bodies a risk for a hot spots (Clark et al., 2008, Oesterholt et al., 2006).

In camp 3 a *P. vivax* hot spot was detected in the same area by using all the three measures (RDT, PCR and serology). The hot spot comprised of households most of which were under the bracket of lower SES, and reported both limited number of ITN ownership and its use the night prior to the survey (data is not shown). These household were also located in between two river branches, which may associated with the high levels of vivax malaria. Only one hot spot was detected for *P. falciparum* by using age adjusted ODs data, which is located near to *P. vivax* hot spot but at different position. In camp 4 one hot spot of age adjusted antibody responses against

P. vivax was found. In camp 5 only one hot spot of age adjusted OD against *P. falciparum* was detected, while two cold spots *P. vivax* seroprevalence were detected, consisted of households located close to BHU and away from the rainwater runoff.

Variation in a disease risk (geographical clusters of disease incidence) within a village/area, which is genetically homogeneous, may be because of variation in exposure to malaria-infected mosquitoes (Bousema et al., 2010a), which is consistent with the finding of this study as all camps were situated nearby mosquitoes breeding sites. Households were scattered along water bodies in three camps (Camp 1 and camp 3 along rivers and camp 5 along canal) and camp 2 and 4 are surrounded by rice and sugarcane fields which have standing water most of the year (Aerial views of camps captured from Google earth).

In several studies carried out in areas of higher endemicity, distance to water bodies was associated with malaria incidence and immune responses (Clark et al., 2008, Oesterholt et al., 2006, Staedke et al., 2003, Kreuels et al., 2008, Wilson et al., 2007, Creasey et al., 2004). Another study also described that proximity to the mosquito breeding sites is not the only predictor of malaria risk, due to variation in the breeding sites productivity and longevity (Fillinger et al., 2009). However, this is not always true as one study conducted by Bousema et al. (Bousema et al., 2010a) in Somaliland found no association of malaria incidence and distance to water body. Detectable water bodies may also be misleading as mosquitoes breeding sites may be quite small and transitory and likely to be missed during surveys. For example small burrow pits made by horse or cattle or any other animal tracks, dirt road or dirt tracks, tyres, buckets and open water tanks which may be a potential breeding habitat for mosquitoes. This survey did not identify or collect information about mosquitoes breeding sites in the camps, which may be one of the reasons of these households to be in the hot spot. Therefore it will be useful for control programmes to check for potential mosquitoes breeding sites and might apply vector control measures to target outdoor biting vectors.

Household factors (Greenwood, 1989, Clark et al., 2008, Oesterholt et al., 2006, Kreuels et al., 2008, Gamage-Mendis et al., 1991) and the vicinity of alternative hosts (cattle) have also be associated with hot spots of malaria incidence (Bousema et al., 2010a). Which is what was found in this study, where high risk of infection and seroprevalence to *P. vivax* were associated with low SES and poorly built houses (mud houses with Thatched roofs and living rooms with open eaves), sleeping with animals in the same courtyard and traveling of individuals to other malaria endemic areas (discussed in chapter 5).

Other factors which were identified as being associated to living in a hot spot of malaria transmission included, distance to the nearest breeding site (Clark et al., 2008, Oesterholt et al., 2006, Kreuels et al., 2008), bed net coverage (Clark et al., 2008) and walling material (Somi et al., 2007), which are considered as a key factors in determining the risk of malaria at individuals level. It is important for malaria elimination to identify hot spots of malaria transmission within or among the areas and the risk factors associated with these hot spots. Emigration of asymptomatic parasite carriers or transportation of infectious mosquitoes can lead to spread of parasites beyond the borders of a focus. Therefore it may be useful to improve the housing condition, to create malaria awareness about the risks involved with increase in malaria burden and to screen individuals before leaving or entering in to the hot spot.

To reduce high exposure to mosquitoes and high prevalence of parasite carriage (asymptomatic) in the hot spot both human and vector hosts control interventions will be required as previously suggested (Bousema et al., 2012). Scaling up conventional vector control tools such as LLINs and IRS may be useful for controlling malaria in these hotspots. In addition to these indoor biting and resting malaria vectors control measures it will be potentially useful to target outdoor biting vectors (Reddy et al., 2011, Russell et al., 2011) for example larviciding of mosquito breeding sites and adult vector (Fillinger et al., 2008). However, widespread adoption of such operational constraints has not been encouraged, but they are suggested to be utilized as a targeting strategy.

For reduction of malaria transmission in hotspots it is equally important to control the human infectious reservoir (symptomatic and asymptomatic parasite carriers)

(Stresman et al., 2010). Therefore, it may be useful to treat households and neighbours of individuals who are diagnosed with malaria at health facilities (Moonen et al., 2010) and screen people in the hotspots for parasitaemia at regular intervals (Moonen et al., 2010). Moreover to clear infections in symptomatic and asymptomatic parasite carriers (Okell et al., 2009) mass drug administration (MDA) was suggested where a full therapeutic antimalarial drug dose are administered to a population without prior screening (Okell et al., 2011, Bousema and Drakeley, 2011).

Chapter Seven

Prevalence and molecular characterization of G6PD deficiency in Afghan refugees, KP, Pakistan

Chapter 7. Prevalence and molecular characterization of G6PD deficiency in Afghan refugees, KP, Pakistan

7.1. Introduction

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common red blood cell inherited enzymopathy which affects about 400 million people worldwide (Cappellini and Fiorelli, 2008) and its global prevalence is approximately 4.9%. It is an X-linked recessive hereditary defect caused by G6PD gene mutations, resulting in abnormally low levels of the enzyme Glucose-6-phosphate dehydrogenase (Beutler, 1992). The enzyme plays an important role in the pentose phosphate pathway, to provide reducing power to all cells in the form of NADPH (reduced form of nicotinamide adenine dinucleotide phosphate), which help cells to compensate oxidative stress that can be generated by several oxidant agents (Cappellini and Fiorelli, 2008). RBCs lack mitochondria and so NADPH from the pentose phosphate pathway is their only means to generate NADPH and thus to prevent any oxidative damage resulting in hemolysis (Luzzatto et al., 2001, Williams, 2006). Thus hemolysis occurs in hemizygotes (heterozygous females) and homozygotes on oxidative challenge such as drugs, fava beans and infections. Heterozygous females may be completely asymptomatic or at-risk of hemolysis depending on their lyonization (X-inactivation). In neonates, G6PD deficiency may cause hyperbilirubinemia.

Clinically, G6PD deficiency is characterized on the basis of residual activity of Glucose-6-phosphate dehydrogenase enzyme. Enzyme activity ranges from its complete absence (classified as Class I) to abnormally high levels (classified as Class V with enzyme activity of >150%) (Beutler, 1994). Several studies have shown that G6PD deficiency has a protective action against malaria (Frank, 2005, WHO, 1989, Ruwende and Hill, 1998). The global distribution of G6PD deficiency is very similar to that of malaria with a high prevalence of both in the tropics and subtropics. In malaria patients however, severe G6PD deficiency can complicate the infection and treatment with some antimalarials like Primaquine or Dapsone can lead to fatal intravascular hemolysis leading to acute renal failure (Wickramasinghe and Abdalla, 2000).

Frank et al., (2005) asserted on prescription of G6PD deficiency tests for patients that experience acute hemolytic reaction on exposure to oxidative drugs or when they

have a family history other underlying systemic disorders for example of jaundice, splenomegaly, or gallstone disease (Frank, 2005). These conditions should be particularly considered among populations of African, Mediterranean, or Asian origin (Minucci et al., 2008).

The accurate diagnosis, however, is variable depending on the stage of the diagnosis or whether the patient is a male or female (Minucci et al., 2008). Some commonly used semi-quantitative assays such as the fluorescent spot tests can be used in countries where the deficiency is both frequent and is endemic to malaria. Since the test is inexpensive, rapid and sensitive, it can be used in screening large scale populations. Other similar tests include the Heinz body examination and GSH stability test (Tantular and Kawamoto, 2003). However, in cases of definitive biochemical identification (levels of enzyme concentrations) a quantitative assay may be required. The commonly used spectrophotometric assay measures the rate of NADPH generation at wavelength of 340 nm by adding a precise amount of hemolysate to the substrate of glucose-6-phosphate and its cofactor NADP (Beutler, 1984). However, the test is reportedly prone to producing false negatives when performed soon after a hemolytic episode (Ringelhahn, 1972).

The genetic variation in the heterozygous females (especially due to X-inactivation) affects the clinical outcome of the deficiency, such that these females might show enzyme activity varying from hemizygote-like phenotype to being normal (Minucci et al., 2008). In such cases quantitative and qualitative testing described above can give counterfeiting results (Peters and Van Noorden, 2009).

Varieties of molecular techniques have been described that detect G6PD deficiency causing mutations even in HF (Du et al., 1999, Zhang et al., 2005, Farez-Vidal et al., 2008, Minucci et al., 2008). About 140 mutations have been reported in the coding regions of G6PD gene resulting in over 450 G6PD variants of which 300 have been acknowledged by WHO. (Town et al., 1992, Hirono et al., 2002, Chen et al., 1991, WHO, 1991). PCR followed by sequencing of the whole or fragment of gene can provide a definite diagnosis and also help in identification of novel and less frequent mutations (Minucci et al., 2008). The technique, however, is expensive and thus not practical for prompt diagnosis especially in field settings. RFLP of the gene with

respect to the known mutations reported in the study population is a rapid and cheap PCR screening approach.

One of the most widely distributed variant of G6PD deficiency is the Mediterranean type (G6PD 563C-T) that causes class II syndrome (1–10% enzyme activity). This type is prevalent in the Mediterranean countries Middle Eastern, and South Asian countries including Pakistan (Luzzatto et al., 2001, Kurdi-Haidar et al., 1990, Karimi et al., 2003, Oppenheim et al., 1993, Bayoumi et al., 1996). In Pakistan comparatively higher prevalence of Mediterranean type has been reported in Pathans with respect to other ethnicities (Punjabi). Some molecular level work in Pakistan has reported 563C-T, silent mutation of 1311 C-T, while 1003A-G and 131C-G was found less-frequent and a novel 973 G-A variant (Moiz et al., 2009, Moiz et al., 2011, Saha et al., 1994). Studies focusing on G6PD deficiency screening before administering anti-malarials [such as Primaquin] have reported 2% to 10% (Brooker et al., 2006) prevalence in Afghanistan.

Plasmodium vivax is the dominant species in the study area, responsible for 70-90% of cases and the remainder of cases are caused by *P. falciparum* (Rowland and Nosten, 2001, Rowland, 1999a, Rowland et al., 1994). *P. vivax* infections cause majority of malaria episodes, which is the most stable species due to its ability of formation of hypnozoites in the liver. For example previous studies conducted in Afghan refugees has shown that one initial infection can cause up to 10 or more subsequent episodes even after effective treatment for acute episodes (Leslie et al., 2008, Leslie et al., 2004). The liver stage hypnozoites act as a reservoir of the disease that may help the disease to continue its transmission cycle. Furthermore, infected individuals with the latent liver stage may reduce the protective efficacy of commonly used preventative measures (insecticide treated bred nets) due to its ability to develop further episodes even if they regularly use an ITN (Leslie et al., 2010, Leslie et al., 2008).

However it is found difficult to control vivax malaria because of the occurrence of relapse episodes. The radical cure for latent stage vivax malaria, is primaquine or other anti-hyozoite drugs but the high prevalence of G6PD deficiency and the lack of G6PD testing facilities among the studied population (Ali et al., 2005, Bouma, 1995) prevents its use (Bouma, 1995).

For an immediate reduction of vivax malaria morbidity and to drop its transmission levels it is essential to make G6PD testing readily available. The aim of this chapter was to measure the molecular prevalence of SNP's associated with G6PD deficiency in five Afghan refugee camps to provide information to aid decisions related to the prescribing of anti-malarial therapy with drugs like primaquine.

7.2. Materials and Methods

505 samples from camp3 were assayed using RFLP PCR (Moiz et al., 2011), to identify G6PD mutations in the study population. The same DNA template was used as that extracted for the identification of low-density parasite infection. The method for extraction is described in detail in chapter 2 section 3.

Initially three mutations [(1003A-G/335 Ala→Thr, Class III), (563C-T/188 Ser→Phe, Class II) and (1311 C-G /44 Ala→Gly, Class III)] (Table 6.1) were selected. However, due to time and financial constraints, only G6PD 563C-T variant was studied. G6PD 563C-T was selected because there is evidence that suggests that it is the most prevalent mutation among Pathans, due to their ancestral origin from the Mediterranean basin (Bouma, 1995, Dupree, 1973). Furthermore G6PD 563C-T belongs to class II mutations, which is associated with severe enzyme deficiency (with less than 10% enzyme activity) as compared to 1003A-G and 131C-G mutations which are class III (Moderately deficient, 10–60% residual activity) (Beutler, 1992).

Table 7.1 Primers and restriction enzymes for various G6PD variants

G6PD variant /aminoacid substitution	REF	Primers	Enzymes	Digested products (Base pairs)
563C-T [type II] /188 Ser→Phe	(Saha et al., 1994, Moiz et al., 2011)	5' ACTCCCCGAAGAGGGGTTCAAGG 3' 5' CCAGCCTCCCAGGAGAGAGGAAG 3'	<i>Mbo</i> II (Fermentas)	377,119 (wild) 277,119,110(homozygous mutant) 377,277,119,110 (heterozygous mutant)
1003A-G [type III] /335 Ala→Thr	(Noori-Daloi MR, 2006)	5' CAAGAAGCCCATTCTCTCCCT 3' 5'TTCTCCACATAGAGGAGGACGGCTGCCAAAGT 3'	<i>Bst</i> XI	130,78 (wild) 100,78,30(homozygous mutant) 130,100,78,30 (heterozygous mutant)
131 C-G [type III] /44 Ala→Gly	(Kaeda et al., 1995)	5' CAGCCACTTCTAACCACACACCT 3' 5' CCGAAGTTGGCCATGCTGGG 3'	<i>Hae</i> III	107,75,66,48,45,11(wild) 123,107,66,45,11(homozygous mutant) 123,107,66, 75, 48, 45,11 (heterozygous mutant)

7.3. Results

7.3.1. Wild and mutant types of 563C-T

Sequencing of 5 samples from camp3 showed digestion product sizes that were comparable to those previously reported (Table 7.1 and 7.2).

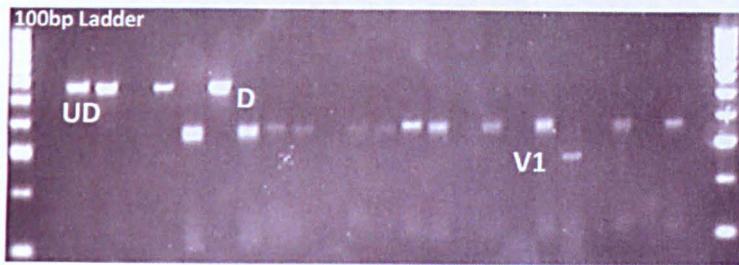
Table 7.2 G6PD wild and mutant type RFLP products after digestion by MbolI of G6PD sequence from this study. Extractor version 1.1.(Stothard, 2006).

Phenotype	Digested products (size in Base pairs)
Wild type	374, 120
Homozygous mutant	276, 120, 98
Heterozygous mutant	98, 120, 276, 374

7.3.2. G6PD deficiency in camp3

The X-chromosomes of 505 subjects belonging to camp 3 for Afghan refugees were analyzed for 563C-T polymorphism of G6PD gene. Of them 563 C-T mutation was observed in only 2 unrelated individuals making (0.4% prevalence). One was male (hemizygous) and another female (Heterozygous). Their ages were 5 and 4 years respectively at the time of diagnosis. The female was negative for malaria by RDT, PCR and serological tests, whereas the male was positive for all the 3 tests.

A.



B.

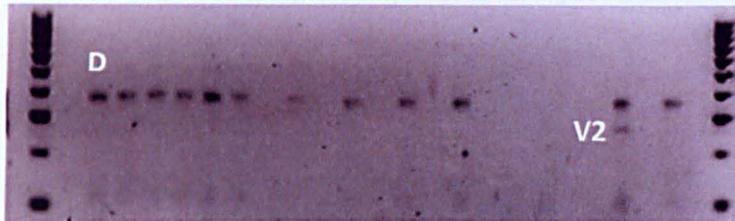


Figure 7.1G6PD PCR and RFLP products.

Key to figures 7.1 A and B: UD=Undigested PCR product, D= digested product wild type, V1=563 C-T variant in male, V2=563 C-T variant in heterozygous female.

7.4. Discussion

The present study originally aimed at identifying 3 mutations (Table 6.1). However, due to time constraints only one (Mediterranean type 563C-T which is WHO class II with <5% enzyme activity) was selected based on its higher reported prevalence in Asian and Middle Eastern populations (Cappellini and Fiorelli, 2008). Secondly, G6PD-Med (belong to the class II) was previously suggested the most likely type prevalent in Pathans, due to origin of Pathans from the Mediterranean basin (Bouma, 1995, Dupree, 1973). Historically, Pathans are suggested to be the descendants of European soldiers who accompanied Alexander the Great (Firasat et al., 2007, Papiha SS, 1999) and are also considered to be the lost tribe of Jews (Papiha SS, 1999), who have high prevalence of G6PD deficiency (Moiz et al., 2009).

A prevalence of 0.4% was observed camp 3 of Afghan refugees in the present study. A comparatively higher frequency of G6PD deficiency has been previously noted in Afghani is ranging from 2% to 10% (Brooker et al., 2006). Bournal et al determined the prevalence of G6PD deficiency in Afghan Safi tribe to be 15.8% as compared 7% in Mohmands (a native Pathan tribe in Pakistan). This difference in prevalence might be a result of lower sensitivity and limitations of RFLP for this specific mutation (563C-T) in the present study. In addition, the present study only typed a single mutation, whereas other studies that report higher figures of G6PD deficiency among Afghans are based on the disease's clinical diagnosis which may encompass a much wider range of mutations.

A few molecular level studies in Pakistan have reported 563C-T, silent mutation of 1311 C-T, 1003A-G, 131C-G and a novel 973 G-A mutations. These studies showed that 563C-T mutation was more common in Pathan patients as compared to other ethnicities (Moiz et al., 2009, Moiz et al., 2011, Saha et al., 1994). These RFLP based studies in Pakistan to detect a specific mutation require prior knowledge regarding the mutation's prevalence in the study area. This limits these studies from identifying novel mutations which might confer phenotypic effects. Sequencing G6PD targets can elaborate the knowledge of genetic variants in the region.

Following reports of G6PD deficiency found in Pathans, the use of primaquine for the treatment of *P. vivax* was suggested no longer to be recommended (Bouma M.J. et al.,

1995). Another study conducted in Afghan refugees had also shown that 8 week regimen of primaquine could not be readily used in this population (Leslie et al., 2008, Leslie et al., 2004). However, despite reports of CQ resistance from other parts of Asia, Leslie et al., reported that CQ was still a highly effective drug against vivax malaria, when given as the standard 14-day course (Leslie et al., 2008, Leslie et al., 2004, Leslie et al., 2007).

General Discussion

Chapter 8. General Discussion

The aim of this PhD study was to generate current information on malaria infection through parasite prevalence and malaria exposure using antimalarial antibody responses and to compare the level of malaria transmission intensity among the study camps. RDT, PCR and serology were employed together to detect any likely source of ongoing transmission in this low transmission area and to validate usefulness of serology, PCR and RDT as a tool to monitor malaria endemicity. Since all the three measures have their own advantages and disadvantages and contribute essential information about transmission dynamics especially in low malaria endemic areas. The study will provide additional insight into nature and role of anti-malarial immunity in residents of the study area.

The study also aimed to characterize the risk factors for exposure and infection to both *P. falciparum* and *P. vivax* malaria and to assess its relationship with infection and exposure to malaria in refugee camps in KP to assist decision-makers in better targeting malaria intervention and control efforts in the area. These individual and household risk factors were associated with the increase or decrease of the number of malaria infections and seroprevalence. Additionally GIS data was combined with measures of exposure and infection to map potential foci of malaria infection and exposure to aid malaria control. Variation in the risk of malaria within camps can be used for targeted interventions to identify hot spots of malaria transmission. 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.

Entomological monitoring activities (abundance of vector species, mapping of risk areas and monitoring resistance of vectors to insecticides) and specific parasitological surveys (blood slides or rapid diagnostic tests) are commonly used as direct measurements of potential local transmission. WHO also recommended sero-epidemiological measures which can help in validation of the interruption of local transmission using along with parasitological and entomological surveys (WHO., 2007b). Previous study also used serological measures (SCR and seroprevalence) in combination with parasite prevalence (RDT) to monitor the progress of malaria control interventions on Bioko Island and a reduction in transmission was detected (Cook et al., 2011). Similarly to evaluate transmission dynamic before and after malaria elimination in Vanuatu, serological measure in combination with PR data (RDT and PCR) were used and confirm its progress towards malaria elimination.

Several national malaria programmes acquired elimination strategies in the past 15 years and some of them have succeeded in achieving or nearing that goal. For example, Maldives, Tunisia, and the United Arab Emirates (UAE) most recently eliminated malaria. This successes was made possible due to intense national commitment to achieving zero incidence of infection, together with coordinated efforts by partners and keeping a good record of malaria situation (strengthening the malaria surveillance system) (WHO., 2007a). Iran also adopted national malaria elimination programme in 2005, where a significant change in malaria situation has been observed (the number of malaria cases dropped to 2700 (2011) from 16,000 cases in 2006) by implementing focused interventions and strengthening the malaria surveillance system and applying a participatory approach to include the local communities (UNDP., 2013).

In general, transmission of malaria is limited in KP but since the province is bordering Afghanistan from the west, it has become the host of millions of Afghan refugees from the endemic malarial areas of Afghanistan. Malaria was widespread in refugee camps in 1990s but nowadays as a result of the anti-malaria campaign started in late 1990s after epidemic in refugee camps, malaria transmission was interrupted. Previous published (Rowland et al., 2002 b) and BHU data from the camps showed that the rate of malaria was steadily declined since past decades after epidemic in the refugee camps in late 1990s. The decline observed in malaria transmission in most of the

Afghan refugee camps are suggested to be due to the introduction of intensive malaria control measures. But other factors such as socio-economic development are likely to be involved in the decline. Therefore this study was designed to facilitate the control programmes to assess the presence of the parasite carriers and to evaluate the reservoir of infections by using RDT and nested-PCR, where transmission is perceived to be reduced in the last decade. The low prevalence of malaria parasites in the studied area show malaria can be control and ultimately eliminate in the near future if to establish a proper surveillance and follow up system and the level of transmission needs to be continually monitored. Very limited and old published data is available on malaria situation in the study area and accurate data on the proportion of malaria cases and population at risk are essential for the design and implementation of new interventions. So the current data on the malaria burden at population level will help to accurately update malaria situation and will provide additional information on the population at risk, which can help malaria control activities and guide research in the study area.

8.1. Malaria infec on through parasite prevalence

In the study no *Plasmodium falciparum* parasites were detected by RDT but using PCR 7/506 (1.8%) in camp3 and 1/132 (0.8%) in camp 4 positive individuals were detected. Low levels of *P. falciparum* prevalence detected by the study are in agreement with BHU and previous published data that also reported decline and extremely low *P. falciparum* infection prevalence in the studied camps. Other countries who also reported reducing asymptomatic falciparum malaria includes Iran (Zoghi et al., 2012), Sri Lanka (Fernando et al., 2009) and Kenya (John et al., 2009). The reduction observed in the *P. falciparum* infection prevalence and asymptomatic individuals in these refugee camps in the past and in the above mentioned countries was thought to be due to applying malaria control interventions firmly (Rowland et al., 2002 b).

Data from the surveys showed overall 4% (96/2522) *P. vivax* parasite prevalence detected by RDT at all study sites with the highest 9% prevalence in camp 3 and lowest in camp 1 (0.4%). This low parasite prevalence corresponds with data from the BHU collected in the past decade. Overall prevalence of *P. vivax* infection detected by PCR was 12% with a range of 5-15%. Camp 3 with high prevalence of infection detected by RDT and camp 1 the lowest among the camps. *P. vivax* parasite carriage measured by

PCR is approximately 4 times greater than measured by RDT, which suggest the presence of asymptomatic carriers (carriage of low levels of parasites without symptoms) or sub-RDT (infection below the sensitivity of RDT but picked up by PCR) in the study area. In endemic areas asymptomatic carriers of malaria are a result of continuous exposure, 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 submicroscopic phase, while in high-transmission areas repeated infections and superinfection may results in the increase of average parasite density in an infected individuals (Okell et al., 2012).

Other low endemic areas that also reported submicroscopic infections of malaria have included, 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) and Peru (Roper et al., 2000) and Principe (Lee et al., 2010). High transmission areas including Nigeria (Eke RA, 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 A, 2008), Gabon (Klein Klouwenberg et al., 2005, Nkoghe et al., 2011) and intermediate transmission areas India, Burma (Richards et al., 2007), Thailand (Coleman et al., 2002) also reported the presence of asymptomatic carriers in the population. It has been observed that when assessed by Pfs25 QT-NASBA, gametocyte prevalence amongst parasite carriers may be as high as 90% (Bousema et al., 2006). Similarly, studies previously published comparing PCR and microscopy for *P. falciparum* have shown the high sensitivity of PCR as compared to microscopy (Okell et al., 2009, Okell et al., 2012).

All these studies mentioned above have shown that microscopy and RDT can miss considerable number of individuals with low-density parasitaemia and is considered as a great challenge for malaria control and elimination programmes. Because individuals with submicroscopic infections can act as carriers of malaria transmission through gametocytes thus can hinder with elimination and control strategies (Shekalaghe et al., 2007, Sutherland et al., 2007, Roper et al., 1996). Schneider et al. have also suggested that submicroscopic infections of malaria can contribute in a similar way as microscopic gametocytes do (Schneider et al., 2007). Therefore, survey on the

prevalence of asymptomatic individuals in diverse malaria settings has been suggested for the successful elimination and final eradication of malaria, is recommended.

8.2. Malaria exposure using anti malarial antibody responses

In chapter 4 serological markers were used to assess malaria exposure and to examine heterogeneity in malaria transmission in the five Afghan refugee camps. Several studies used serological data to assess changes in transmission intensity (Stewart et al., 2009). As previously serological markers were used to detect heterogeneity in malaria transmission in low endemic district of Somalia (where none of the slide microscopy or RDT showed parasite carriage in the population) (Bousema et al., 2010c).

Antibody responses against *P. vivax* and *P. falciparum* were seen in 28% and 6% of respectively the total examined individuals. Overall seroprevalence of antibody against *P. falciparum* antigens was low with no RDT positive and with extremely low overall PCR prevalence (0.4%). Possible explanation for the detection of a very low seroprevalence of *P. falciparum* among examined subjects may be the extensive use of malaria control interventions in the camps since late 1990s after epidemics observed in the refugee camps (Rowland et al., 2002 b).

Statistically significant ($p < 0.05$) seroprevalence (to both *P. falciparum* and *P. vivax*) differences were observed among the camps, which is evidence of heterogeneity in malaria exposure among the camps. Seroprevalence of *P. vivax* increased with age, which suggested exposure-driven age acquisition of antibody response. SCR estimates also demonstrate that there is significant heterogeneity in transmission across the study site and the current upper 95% confidence limit (upper estimates of transmission) for *P. vivax* ranges from 0.02-0.07 and for *P. falciparum* ranges from 0.001-0.01. These results suggest that, despite of reduction in malaria transmission in the studied camps in the past two decades (Rowland et al., 2002 b) the impact of malaria control is uneven, demonstrated by differences in the current force of infection of the camps.

This is the first study to determine asymptomatic infections and exposure to malaria in five Afghan refugee camps in KP, Pakistan. There is no available data for prevalence of asymptomatic infections and exposure to malaria from this area with comparable

epidemiology. Since this is the first sero-prevalence survey in the study area using recombinant MSP-1₁₉ and AMA-1, therefore direct comparison to previous studies is not possible.

Although seroprevalence is a cumulative measure of exposure over time and less prone to brief fluctuations in transmission, therefore the differences in the seroprevalence among the camps described here may indeed replicate a relatively stable pattern of malaria transmission. As in the study area malaria transmission is highly seasonal and serology will be valuable tool to assess transmission, as it is less dependent on the timing of the survey than direct assessment of parasite burden by PCR or microscopy that can only provide a snapshot of transmission.

Cross reactivity between immune responses to malaria and other parasites have been reported previously (Abramo et al., 1995, Naus et al., 2003) but these were suggested less pronounced when recombinant proteins were used instead of whole parasite extract. An alternative approach to reduce the likelihood of cross-reactive antibody responses speculated was to use sera at a minimum dilution of 1:80 (Abramo et al., 1995). Serum dilution used (to test the samples) for this study was 1:1000 samples (considerably higher dilutions) and no relation in antibody titers between the homologous antigens of *P. falciparum* and *P. vivax* observed (detailed in chapter 4). Furthermore, to calculate cutoff value to decide seropositivity, seronegative population was derived from within the same samples, in order to elimination false-positive results and to reliably detect low-level local malaria transmission.

Moreover, seropositivity to malarial antigens may not necessarily represent recent exposure in the study area (Corran et al., 2007, Warren et al., 1976), due to longevity of antibody responses. For example in adults, previous exposure to parasites in life or travel to malaria-endemic areas in the past can make immune responses doubtful resulting from recent local transmission (Warren et al., 1975). Once acquired, antibody responses against MSP-1₁₉ and AMA-1 are suggested to persist for several years (Drakeley et al., 2005). For this seroprevalence in younger age groups was used to investigate current malaria transmission. Low seroprevalence against *P. falciparum* are also in line with the drop in *falciparum* parasite prevalence and a reduction in *P. falciparum* prevalence reported by BHU.

SCR estimates were examined for associations with prevalence of infection measured by PCR and RDT on camp level. These results show a good association between SCR and prevalence of infection by both RDT and PCR. These data is in line with the results from a study, where an association was also found between SCR and parasite rates (Cook, 2010). SCR rates were also related to EIR, to estimate *P. falciparum* transmission intensity and good correlation was between the two mentioned measures (Corran et al., 2007, Bousema et al., 2010c).

8.3. Risk factors of malaria in study area

Results of chapter 5 aimed to provide information on the potential risk factors involved in exposure and infection of malaria in the study area. It is expected that these results will prove useful in assessing potential malaria risk factors and improve the malaria control programs targeting of interventions.

In these surveys I found that all age groups were exposed to *P. vivax* infections evidenced by antibody responses, but a high proportion of parasite positive individuals were observed amongst the young (1-5) and middle age (6-20) groups, which was in line with the situation of malaria in a low endemic areas in India (Ramachandran, 2010).

Serological data for both *P. vivax* and *P. falciparum* showed age-dependency in antibody responses; an expected result of the acquisition of antibody response with increasing age due to repeated exposure (Corran et al., 2007, Bousema et al., 2010c, Cook, 2010). Where in similar low endemic area in Somaliland malarial antibody responses has been reported high in older age groups, which build up with repeated exposure during childhood.

Travelling history was used to investigate whether movement of local resident to other endemic areas was a risk factor of malaria in the camps. Staying in the camp of resident from the last six months prior to survey was associated with lower risk of both *P. vivax* (RDT/PCR) and *P. falciparum* (antibody responses). Number of other studies also suggested the movement of local resident to other neighboring endemic areas is an important risk factor of malaria in low transmission settings (Danis-Lozano et al., 2007, Osorio et al., 2004, Sornmani et al., 1983, Fungladda et al., 1987, Ramachandran,

2010, Moore et al., 2004).

There is evidence according to the study that the people who had fever within 24 hours and within two weeks were being parasite positive (*P. vivax*) detected by both RDT and PCR as compared to one that had no fever. It validates that parasite positive individuals have fever because fever is one of the symptoms of malaria.

Low SES was associated with high prevalence of *P. vivax* infection (PCR). There are a number of studies which support the evidence of lower socio-economic factors related to high malaria risk (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, Ayele et al., 2012). A study conducted in Amhara, Oromiya and Southern Nation Nationalities and People (SNNP) regions of Ethiopia also reported open eaves and SES as a significant risk factor of malaria (Ayele et al., 2012). Possible explanation for low SES as a risk factor for the prevalence of *P. vivax* infection is living in a house which are more likely to increase contact with the mosquitoes on the contrary households in high SES a bracket (a cement floor, iron roof, houses constructed of bricks) to limit contact with mosquitoes. Moreover individuals with low SES may be less likely to use antimalarial control measures (to reduce favored access of the mosquito vector to man) due to costs. These results shows thatched roof and non glazed windows non significant which were commonly found significant risk factor in previous studies (Ayele et al., 2012).

To examine that cattle might protect occupants against malaria through zoonophylaxis *P. vivax* sero-prevalence was associated with the animals sleeping in the same house as the study participants. Increased seroprevalence against *P. vivax* in the residents of a household was observed who were sleeping in the same courtyard with the cattle. This is in line with previous studies conducted in Afghan refugees (Bouma and Rowland, 1995) and Ethiopia (Deressa et al., 2007, Graves et al., 2009) had also shown that sharing of house with livestock increased the risk of malaria prevalence. Simple explanation for observing high *P. vivax* seroprevalence among residents of households sleeping with animals in the same place is the increased amount of CO₂ and cattle odours that attracts malaria vectors.

Sleeping under ITN previous night before the survey was associated with lower risk of vivax malaria infection (RDT/PCR). Although the single night usage of ITN before the survey is unlikely to accurately capture the ITN usage over a longer period, but still it may represent the behavior of people towards use of ITN. A household randomized trial of ITN in Afghan refugee camps in KP, Pakistan had reported protection against malaria (Rowland et al., 1996, Rowland et al., 1997b) and top sheets, blankets and Islamic head-body veils treated with permethrine, were considered as a protection against both *P.falciparum* and *P.vivax* (Rowland, 1999b). Similarly, a study in Ethiopia also reported lower malaria Prevalence in mosquito nets users compared to non users (Ayele et al., 2012).

Insecticide residual spraying was associated with reduces malaria infection and exposure but these show no significant association. Possible reasons for IRS being non-significant in this study may be that the spraying was done by a household head that were not a trained spraymen and without supervision. As such they maybe not careful about the choice and quality of insecticide certain vector species are resistant to DDT and organophosphate insecticide or the proper time of IRS which may maximize the effect (Rowland and Nosten, 2001). It may also be due to the fact that at such low coverage no effect on the prevalence of infection and exposure was likely to be observed. This study collected information only about IRS within last 12 months in the household and did not deal with efficacy of it. Therefore this study cannot be directly compared with other studies in these refugee camps in the last decade based on different study design. However, IRS was found significant in previous studies conducted by Rowland et al (Bouma et al., 1995, Rowland et al., 1997a) showed protective efficacy against both *P.falciparum* and *P.vivax* in the Afghan refugee camps. Spraying tents with permethrin was found to lower the risk of *P.falciparum* malaria nomadic Afghan refugees (Bouma et al., 1996).

8.4. Hot spots of malaria exposure and infection within the camps

Heterogeneity in malaria has long been observed between the villages or areas where a small group of people is most heavily or frequently infected as compared to majority of locals with no or very few infections majority (Snow et al., 1988, Greenwood et al., 1987). Such variation in a disease risk within a genetically homogeneous (individuals) area may be due to difference in exposure to malaria-infected mosquitoes (Bousema et al., 2010a). To identify such hot spots (geographically distinct unit where infection and disease are concentrated in a small proportion of individuals) of malaria transmission in an area and the risk factors associated with these hot spots will be useful for an area which is close to attaining malaria elimination and where malaria transmission is likely to be sustained in and by a few focal points. In such areas transmission can be reduced effectively by targeting such hot spots with carefully planned interventions (Bousema et al., 2012).

An examination of the spatial variation in malaria infection and exposure in the five Afghan refugee camps is described in chapter 6. Heterogeneity in infection and seroreactivity for both *P. falciparum* and *P. vivax* was observed within the camps. Four of the camps out of five (except for camp 4 which is situated besides rice fields) are located near to water bodies (rivers, streams and canals). Hotspot of antibody prevalences to *P. vivax* in all age groups in camp 1 comprised of households located very close to water logged rice fields, and away from the BHU. As previous studies had also mentioned that distance to water body is associated to malaria incidence and immune responses (Wilson et al., 2007, Creasey et al., 2004, Clark et al., 2008, Oesterholt et al., 2006, Staedke et al., 2003, Kreuels et al., 2008).

However Bousma et al. found no association of malaria incidence and distance to the near water body (Bousema et al., 2010a). As proximity to the mosquito breeding sites is not the only predictor of malaria risk, due to variation in the breeding sites productivity and longevity (Fillinger et al., 2009), moreover detectable water bodies may be misleading, as mosquito-breeding sites can be quite small (Greenwood, 1989, Clark et al., 2008, Oesterholt et al., 2006, Kreuels et al., 2008, Gamage-Mendis et al., 1991). Other factors which were identified as indicators of living in a hotspot of malaria

transmission included less bed net coverage (Clark et al., 2008), walling material (mud and palm walls) (Somi et al., 2007), and the vicinity of alternative hosts (cattle) (Bouma and Rowland, 1995), which are considered as a key factors in determining the risk of malaria at individuals level.

Hotspots are likely to persist as a source of residual malaria transmission, if not targeted (Bautista et al., 2006, Ernst et al., 2006, Gaudart et al., 2006) and are most likely to play a catalysing role in areas of stable transmission and can make a problem in malaria elimination efforts (Moonen et al., 2010). Therefore interventions targeted at transmission hotspots are relevant and important for malaria control (Moonen et al., 2010, Woolhouse et al., 1997, Smith et al., 2007, Bousema et al., 2012). It has been observed that targeting hotspots with long-lasting insecticide-treated nets (LLINs) and indoor residual spraying (IRS) could lead to malaria elimination (Bousema et al., 2012).

8.5. Prevalence and molecular characterization of G6PD

Plasmodium vivax is the dominant species in the study area (Rowland et al., 1994, Rowland, 1999a, Rowland and Nosten, 2001), which is the most stable due to its ability of formation of hypnozoites in the liver. The hypnozoites act as a reservoir and help to continue *P. vivax* transmission cycle. Therefore the control of *P. vivax* has been suggested difficult as compared to *P. falciparum* due to formation of latent liver stages. The radical cure for latent stage vivax malaria is primaquine but the high prevalence of G6PD deficiency among the studied population as compared to other Pakistani population and the lack of G6PD testing facilities (Bouma, 1995, Ali et al., 2005) prevents its use. (Bouma, 1995). As the X-linked G6PD disorder causes hemolysis in hemizygotes and homozygotes on administration of primaquine.

The prevalence of G6PD deficiency in Afghans has been observed in several studies ranging from 2 to 10%, which had focused only on the testing for G6PD deficiency before administering anti-malarials (e.g. primaquin) in Afghans. For an immediate reduction of vivax malaria morbidity and to drop its transmission levels it is essential to make G6PD testing readily available. So the present study aimed to measure the prevalence and to evaluate the molecular characterization of G6PD deficiency.

Initially three mutations [(1003A-G/335 Ala→Thr, Class III), (563C-T/188 Ser→Phe, Class II) and (1311 C-G /44 Ala→Gly, Class III)] were selected for the study but due to time and financial constraints, only G6PD 563C-T variant was studied. Because G6PD-Med (belong to the class II) was suggested the most likely type prevalent in Pathans, due to origination of Pathans from the Mediterranean basin (Bouma et al., 1995, Dupree, 1973).

505 subjects from camp 3 of Afghan refugees were analyzed for 563C-T polymorphism of G6PD gene. 563 C-T mutation was observed in only 2 unrelated individuals with 0.4% prevalence (one was male of age 5 and one female of age 4). Saha et al. and Moiz et al. also described prevalence of G6PD 563C-T (G6PD Mediterranean) in Afghans (Saha et al., 1994, Moiz et al., 2009, Moiz et al., 2011).

The frequency of G6PD deficiency previously noted in Afghani is ranging from 2% (Bouma, 1995) to 10% (Brooker et al., 2006). Living in an area where *P. vivax* was prevalent for millennia, intermarriage, Pashtuns are thought to be the descendants of European soldiers who accompanied Alexander the Great (Firasat et al., 2007) and are also considered to be the lost tribe of Jews (Papiha SS, 1999) (who have high prevalence of G6PD deficiency) (Moiz et al., 2009), were given as the possible reasons of their higher prevalence of G6PD deficiency as compared to other races in the region.

8.6. Conclusion

To detect small-scale differences in transmission intensity serologic markers could be valuable at levels of malaria transmission that are too low for detection by microscopy, RDT, or entomologic tools. This will be helpful for evaluating malaria control programs in low malaria endemic areas. A correlation was observed by this study between serological markers of exposure to malaria and malaria infection prevalence measured by RDT and PCR, which suggest that serological markers of exposure could guide, targeted malaria control efforts. Both parasitological and serological measures were able to detect spatial variation in infection and exposure to malaria at the micro epidemiological level within the camp, which will be useful for spatial targeting of malaria control efforts. Therefore for more robust interpretation of serological data from low transmission areas (where parasite prevalence indicators are less sensitive), congruence between parasitological and serological measures of exposure is needed

(Satoguina et al., 2009). Despite of the low sensitivity as compared to PCR, microscopy should remain a routine diagnostic tool for malaria due to its high specificity as previously observed (Musalika, 2010) and cost and time effectiveness. However for malaria epidemiological studies in this low endemic area a more sensitive diagnostic tool such PCR is recommended.

In summary, the data presented here suggest 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., 2007b).

The absence of *P. falciparum* infection prevalence detected by RDT, very few asymptomatic carriers detected by PCR and extremely low seroprevalence to both *P. falciparum* antigens among examined individuals support the observations of the limited recent *P. falciparum* transmission in the study area. The data suggest that in these studied refugee camps there is the potential to eliminate falciparum malaria.

Malaria control effectiveness may be improved by identifying foci of ongoing transmission, which can be targeted for more frequent and intensive care. Hot spots of malaria were detected in four of the camps. This survey did not collect information about mosquitoes breeding sites, which may be one of the reasons of these households to be in the hot spot. Therefore it will be useful for control programmes 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). Scaling up conventional vector control tools such as LLINs and IRS may be useful for controlling malaria in these hotspots (Reddy et al., 2011, Russell et al., 2011) and to treat everyone 3 or 4 times a year individuals living in the hot spots observed by this study. Moreover malaria exposure and infection was associated with poor housing condition, travelling outside of the camp and behavior of ITN use. Therefore it may be useful to improve the housing condition, to create malaria awareness about the risks involved with increase in malaria burden and to screen individuals before leaving or entering in to the hot spot.

MIS or other community based survey should be conducted (to see the actual burden and transmission) in other parts of Pakistan especially Baluchistan province and tribal areas of KP, where malaria prevalence has been reported high previously as compared to other provinces or areas of Pakistan. Because this high reported prevalence of malaria is based on hospital records or passive case detection which may be unreliable (discussed in detail in the Introduction chapter) and the data is also outdated. The study area (KP), which has the high illiteracy rate as compared to other provinces (especially Punjab and Sindh) of the country, therefore by awareness about protection from malaria, health seeking behavior, prompt diagnosis and treatment can lead to significantly improvement in malaria control and ultimately elimination.

Appendices

Appendix 1 Ques onnaire and Consent form administered in the studied Afghan refugee camps

Title: Assessment of exposure, infec on and risk for malaria in refugee camps in Khyber Pakhtun Khawa (KP), Pakistan

PI: Sobia Wahid

Date: / /2010

Section 1: Household Questionnaire 2010		
1.1	District: (1=Mardan, 2=Peshawar, 3=Malakand)	district __
1.2	Camp name? 1=Adezai 2=Baghicha 3=Kagan 4=Jalala 5=Zangal patai	Cname __
1.3	No. of households in the compound?	No.hh __
1.4	Household ID	hhid __ _ _
1.5	Who is the respondent? 1=Household head, 2=Representative	whoresp __
1.6	Does the respondent agree? 1=Yes, 2=No	respagree __
1.7	Family size () No. of people in each age group: 0-5() 6-20() > 20()	
1.8	What is the highest level of education completed by Household head? 1=None 2=Primary 3=Secondary 4=Higheir	Hhedu __
1.81	Is there anyone who owns?houshold asset ownership?	
1.82	1= yes 2= no	TV __
1.83	Fridge 0= no 1= yes	Fridge __
1.84	Radio 1= yes 2= no	radio __
1.85	Bicycle 0= no 1= yes	bike __
1.9	Mobile pone 1=yes 2=no	bicycle __
1.10	Is the house connected to electricity? 0= no 1= yes	electric __
1.11	What is the main type of fuel used by your family for cooking? 1=Electricity/gas 2=charcoal 3=kerosene4=dung 5=Wood 6=other	fuel __ otfuel
1.12	Are there ducks or chickens? 0= no 1= yes	__ _ _
1.13	Do you have animals in this household like goat,	__ _ _

	sheep or cattle-how many? 0= no 1= yes			
1.14	What type of wall was used for the construction of this house? 1=clay/mud 3=cement/painted 2=bricks/stones 4=other	__ housecons		
1.15	What kind of toilet facilities does your household have? 1 = Flush toilet 2 = Pit toilet/latrine facility/bush/field 3 = No	__ toilet		
1.16	What is the main material of the roof? 1= Iron sheets or tiles 2= Thatch/grass or leaves (explain) 3= Other	roof __ otroof		
1.17	What is the main material of the floor? 1=eath/sand 3=dung 2=cement 4=carpeted 5=other	roof __ otfloor		
1.18	How many windows are glazed? 0= no 1= yes	windglaze __		
1.19	Are the eaves open or closed? 0=open 1=closed	eaves __		
Sec on 2: Malaria Control / preven on				
2.1	Do you have mosquito bed net? at home? 0= no 1= yes	Net Number: [] []		
2.4	Were these nets hanging last night? 0= no 1= yes	ITN hanging __		
2.8	Is the net observed? 0= no 1= yes	netseen __		
2.9	In the past 12 months, has anyone sprayed the interior walls hhold? 0= no 1= yes	IRS __		
2.1 1	Who sprayed the house?	1=Govt.prog,2=NGO,3=hh oldmember, 4=other(explain) 99=don't know		
2.1 2	Have any of these been used in your house over the last week?	1=Mosquito coils 2=Insecticide spray 3=Repellents 4=other(explain)		
Sec on 3: Individual based ques onnaire				
		Mem1	Mem2	Mem3
3.1	ID number			
3.2	Sex 1= male 2= female			
3.3	Age/date of birth			

3.4	Has you lived here for at least last 6 months' live here? 0= no 1= yes			
3.5	Did you stay here last night? 0= no 1= yes			
3.8	Have you always lived in this district? 0= no 1= yes			
3.9	If NO, which other district have did you live in most recently?			
3.1	Have you travelled outside this camp in the last 3 months? 0= no 1= yes			
3.1	When did you come back from your most recent trip? 1= <two weeks 2= <four weeks 3= >4 weeks			
3.1	Have you been ill with a fever at any time in the last 2 weeks? 0= no 1= yes 2=not sure			
3.1	Did you seek advice or treatment for the fever from any source? 0= no 1= yes			
3.2	Did you have a fever in the last 24 hours? 0= no 1= yes 2=don't know			
3.2	Did you take any drugs in the last 2 weeks? 0= no 1= yes			
3.2	Did you use an ITN last night? 0= no 1= yes			
Malaria/Record the RDT result: 0= Nega ve1= Posi				
GPS: Longitude			Latitude	

Information and Consent Form

Title of Study: Assessment of exposure, infection and risk for malaria in refugee camps in Khyber Pakhtun Khawa (KP), Pakistan

Institution: London School of Hygiene and Tropical Medicine

Principal Investigator: Sobia Wahid

Tel No:

Local Investigator:

Tel No:

The London School of Hygiene and Tropical Medicine, and Peshawar University are doing a survey. The present study is designed to use seroprevalence to identify exposure and the risk factors to both *P. falciparum* and *P. vivax* malaria in KP to guide researchers and decision-makers in targeting intervention efforts. Moreover, the study may provide insight into the nature and role of antimalarial immunity in the targeted population. Additionally by combining GIS data with infection and serological

responses we hope to map potential foci of infection to aid malaria control. Approximately 2500 people will be in the survey.

If you agree to take part in the survey, we will ask you questions about your house. We will ask about what you do to prevent malaria, whether you or your child has had a recent fever and about your recent trips outside of the camps. We will also take a few drops of blood by pricking the finger or heel of three members of your family. We will take approximately 1/10 teaspoon (about ½ ml) of blood: The amount of blood we will take is very small and so no harm will come to anyone who agrees to be in the survey. We will test the blood sample for malaria. There may be a small bruise or temporary mild pain on the finger or heel where the blood is taken. There is also a small chance of infection when blood is drawn. However, our careful procedures make this very unlikely. The RDT is free of charge and after positive infection confirmation by RDT e will refer to basic health unit so they can be checked completely where treatment is free.

You are free to choose for you and your child to be part of this survey. The facts we collect in the survey and the results of the lab tests will be kept private to the extent allowed by law. Your name and your child’s name will not be used on any of the survey reports or survey samples.

Today’s survey: The consent form has been explained to me and I agree to take part in the survey. I understand that I am free to choose for me and my child to be in this survey and that saying “NO” will have no effect on me or my child.	If you agree, circle “YES”
	YES

Adult/mature minor/Parent/guardian	Name:	Signature:.....	date □□/□□/□□
---------------------------------------	-------------	-----------------	---------------

If unable to write, please indicate consent with fingerprint

Appendix 2 Prevalence of *P. vivax* infec on across the age group in each camp

<i>P. vivax</i>	Age group	PCR			RDT		
Camp	(years)	Numbr Tested (N)	Number +ve	Percent+ve	Numer Tested (N)	Number +ve	Percent+ve
Adezai	1-5	47	5	10.6	169	1	0.6
	5-20	49	1	2.0	171	1	0.6
	>20	41	4	9.8	167	0	0.0
	Total	137	10	7.3	507	2	0.4
Baghicha	1-5	45	4	8.9	169	5	3.0
	5-20	49	7	14.3	170	5	2.9
	>20	43	8	18.6	168	4	2.4
	Total	137	19	13.9	507	14	2.8
Jalala	1-5	167	21	12.6	167	23	13.8
	5-20	170	41	24.1	170	23	13.5
	>20	168	17	10.1	168	3	1.8
	Total	505	79	15.6	505	49	9.7
Kaghan	1-5	44	3	6.8	161	3	1.9
	5-20	44	2	4.6	168	3	1.8
	>20	42	4	9.5	167	4	2.4
	Total	130	9	6.9	496	10	2.0
Zangal patal	1-5	46	7	15.2	169	6	3.6
	5-20	51	10	19.6	168	10	6.0
	>20	45	6	13.3	170	5	2.9
	Total	142	23	16.2	507	21	4.1
Combined data		1,051	140	13.32	2,522	96	3.81

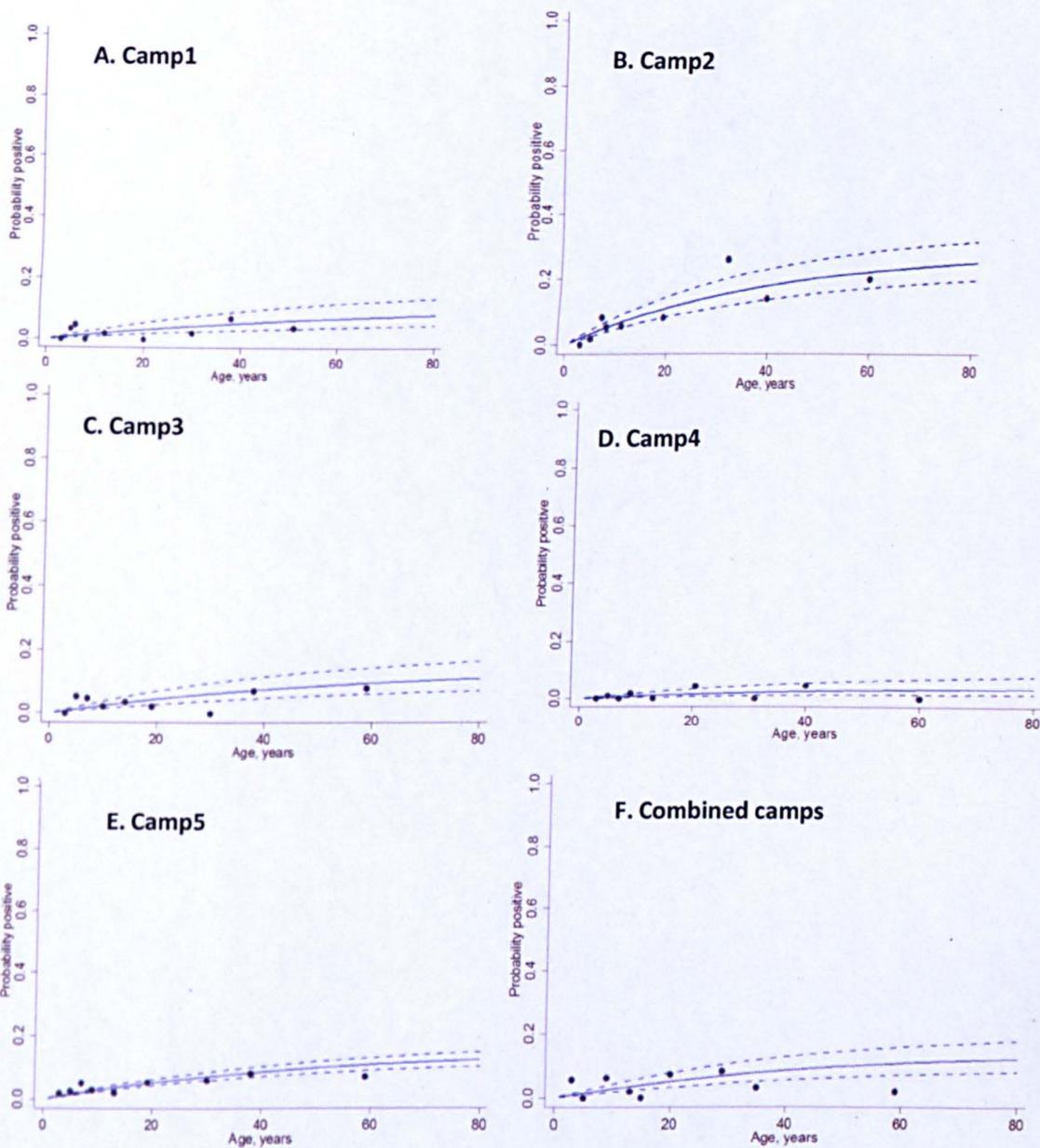
Appendix 3 Seroprevalence to *P. falciparum* gens across the age group in each camp

Camp	Age group	PfAMA-1			PfMSP1 ₁₉		
		Numbr Tested	Number +ve	Percent+ve	Numer Tested	Number +ve	Percent+ve
Adezai	1-5	167	4	2.3(0.5-5.1)	169	0	0
	5-20	168	3	2.3(0.7-5.3)	168	2	1.6(1.1-4.3)
	>20	166	6	4.6(1.0-8.3)	166	1	0.8(0.7-2.3)
	Total	501	13	3.0(1.2-4.8)	506	3	0.8(0.3-1.8)
Baghicha	1-5	168	2	1.6(0.7-4.0)	167	0	0
	5-20	168	9	5.2(1.0-9.4)	168	0	0
	>20	168	35	20.7(14.4-27.1)	161	3	1.6(0.3-3.5)
	Total	504	46	9.0(6.3-11.7)	499	3	0.6(0.1-1.1)
Jalala	1-5	167	6	2.5(0.4-4.7)	166	0	0
	5-20	170	5	2.3(0.2-4.5)	166	4	1.5(0.09-3)
	>20	168	8	4.3(1.2-7.5)	168	0	0
	Total	505	19	3.0(1.5-4.5)	504	4	0.5(0.03-1)
Kaghan	1-5	161	1	0.4(0.4-1.4)	161	0	0
	5-20	168	1	1.5(1.5-4.7)	164	4	1.8(0.1-3.7)
	>20	167	4	2.5(0.1-5.1)	165	2	1.9(0.8-4.7)
	Total	496	6	1.4(0.2-2.6)	496	6	1(0.05-2)
Zangal patal	1-5	169	6	5.7(0.8-10.6)	151	11	5.4(2.0-8.0)
	5-20	168	6	5.4(0.4-10.5)	146	12	9.6(3.8-15)
	>20	170	9	5.3(1.7-8.9)	148	9	4.5(1.2-7.6)
	Total	507	21	5.5(2.1-8.8)	507	32	7(3.6-9.8)
Combined data		2513	105	4.3(3.3-5.3)	2518	48	2(1.0-2.2)

Appendix 4 Seroprevalence to *P. vivax* antigens across the age group

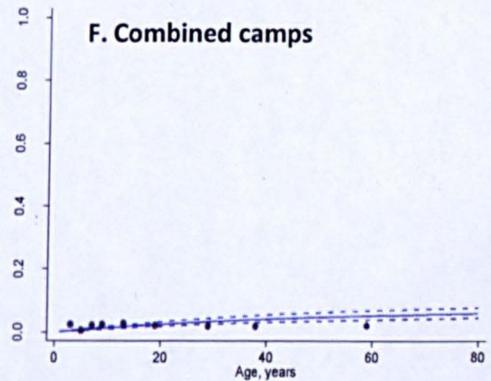
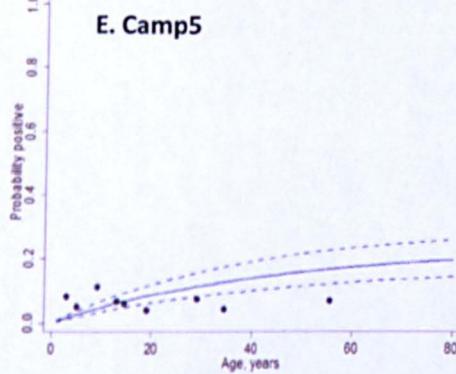
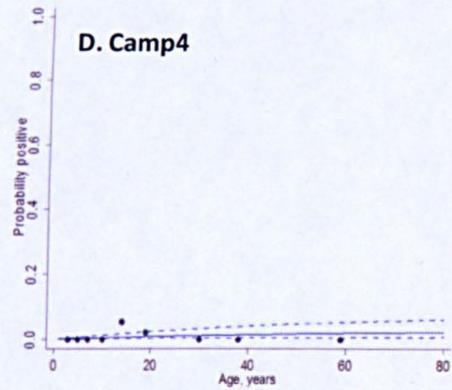
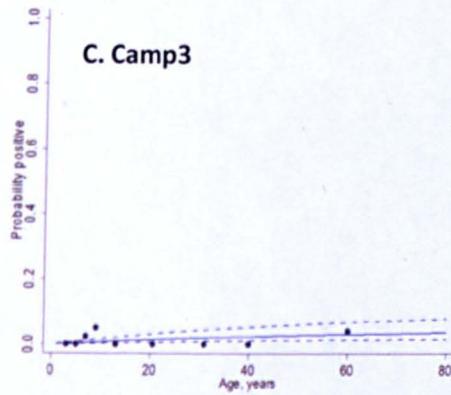
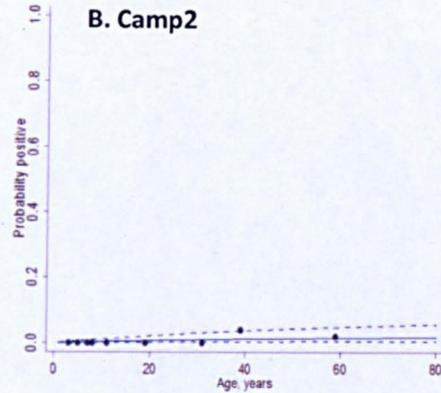
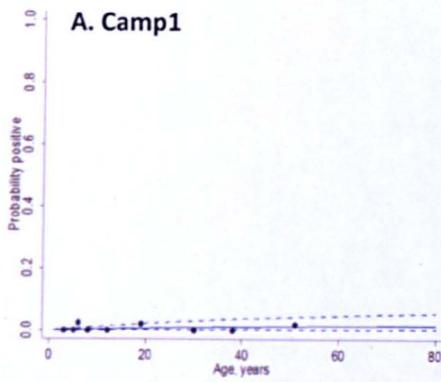
Camp	Age group	PvAMA-1			PvMSP-1 ₁₉		
		years	N.tested	N. +ve	Percent +ve	N.tested	N. +ve
Adezai	1-5	169	5	2.5(0.2-4.9)	169	1	0.4(0.4-1.2)
	5-20	170	18	10.4(4.7-16.2)	170	8	3.2(0.9-5.6)
	>20	162	55	38.2(29.9-46.6)	167	7	4.9(1.3-8.5)
	Total	501	78	15.7(12.1-19.3)	506	16	2.7(1.3-4.0)
Baghicha	1-5	168	7	5.1(1.0-9.1)	167	12	9.4(3.9-14.8)
	5-20	168	28	17.6(10.6-24.6)	168	24	13(7.5-18.5)
	>20	168	111	64.0(56.5-71.6)	168	15	8.6(4.2-12.9)
	Total	504	146	28.3(24.5-32.1)	504	51	10(6.7-13.3)
Jalala	1-5	167	35	17.4(11.4-23.4)	167	51	30(22.3-38.1)
	5-20	170	48	28.6(20.8-36.4)	169	50	27.5(19.8-35)
	>20	168	108	62.9(54.8-71.0)	168	41	24.5(17-31)
	Total	505	191	34.8(30.2-39.4)	505	142	27.6(22.4-32.8)
Kaghan	1-5	161	11	6.7(2.5-11.0)	160	5	2.9(0.23-5.6)
	5-20	167	23	11.5(6.1-17.0)	168	12	6.6(2.6-9.9)
	>20	167	95	57.3(49.4-65.3)	167	12	6.4(2.6-9.9)
	Total	495	129	24.0(20.2-27.9)	496	29	5(3-7)
Zangal patai	1-5	169	4	2.9(0.4-6.3)	169	20	9.1(4.9-13.3)
	5-20	168	11	6.60(2.2-10.9)	167	23	13.2(7.3-19)
	>20	170	31	16.8(10.8-22.8)	170	20	11.4(6.2-16.5)
	Total	507	46	8.2(5.4-11.0)	506	63	11.3(8-14.5)
Combined data		2512	590	23.0(21.3-24.7)	2518	301	11.5(10.0-13.0)

Appendix 5 PfAMA-1 age specific seroprevalence plots in each studied camp of KP A) camp1 B) camp2 C) camp3 D) camp4 E) camp5 F) all camps combined data.



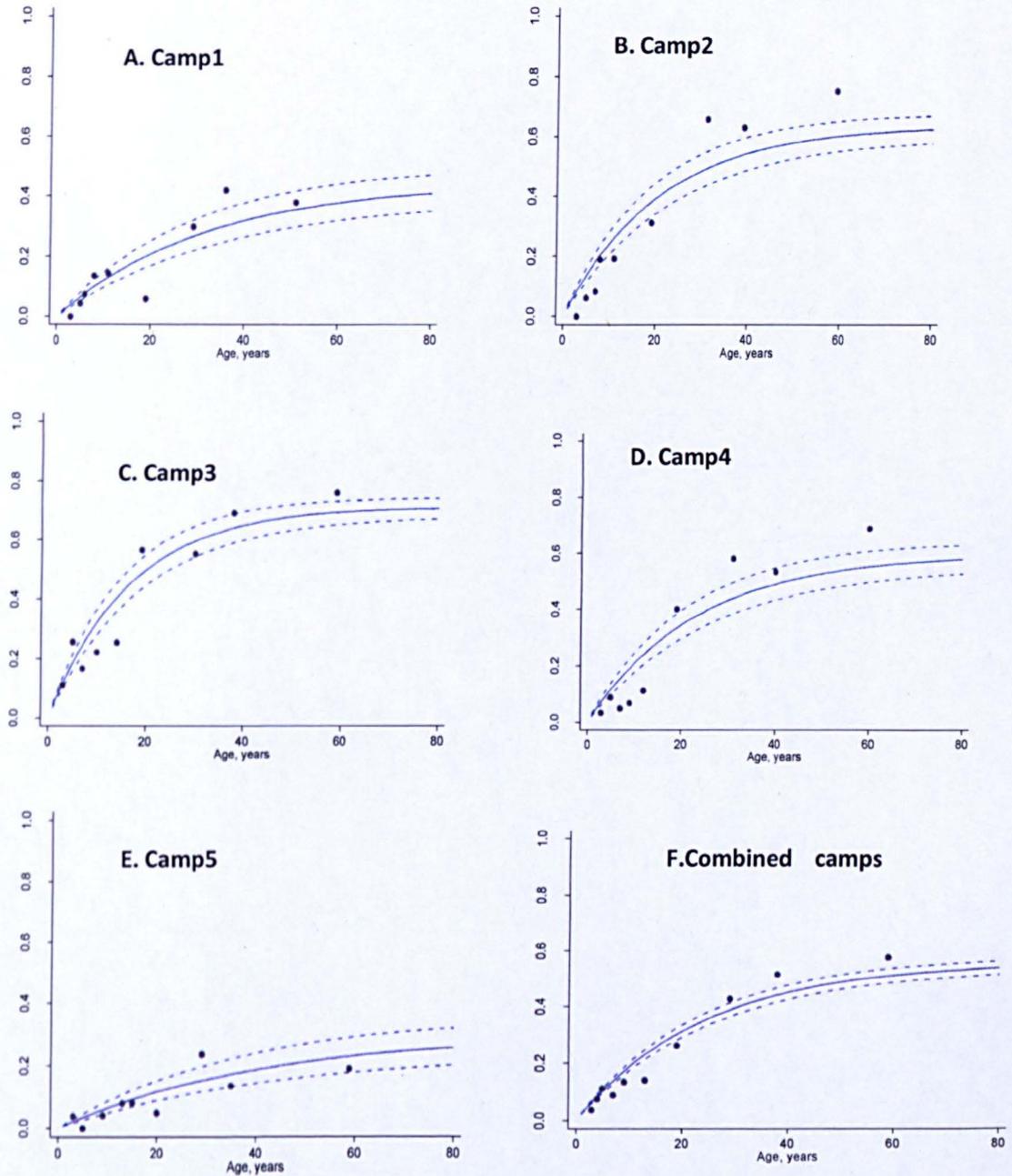
Filled circles represent observed data and the line is the fitted equation 1 in chapter 3 section 3.5.1.3. The proportion of seropositive individuals is represented by the vertical axis in each age group; the horizontal axis is the midpoint age of each age group, while dashed lines indicating 95% confidence intervals by likelihood ratio test.

Appendix 6 PfMSP-1₁₉ age specific seroprevalence plots in each studied camp of KP A) camp1 B) camp2 C) camp3 D) camp4 E) camp5 F) all camps combined data.



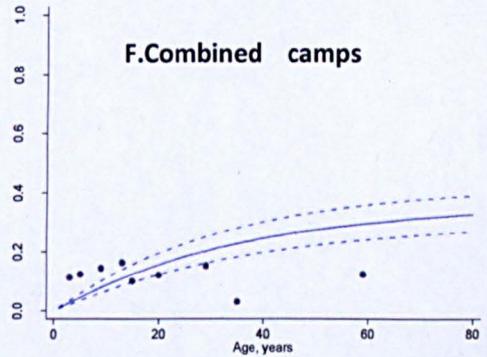
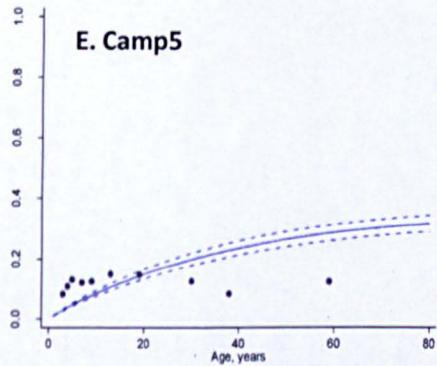
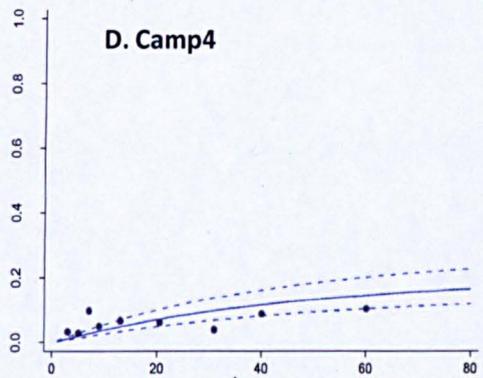
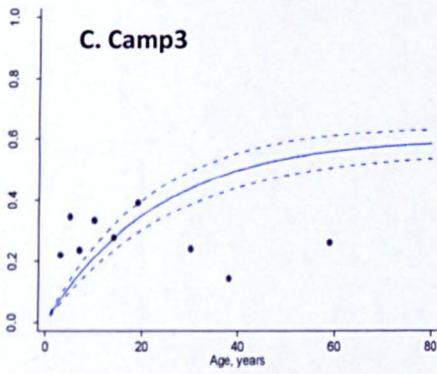
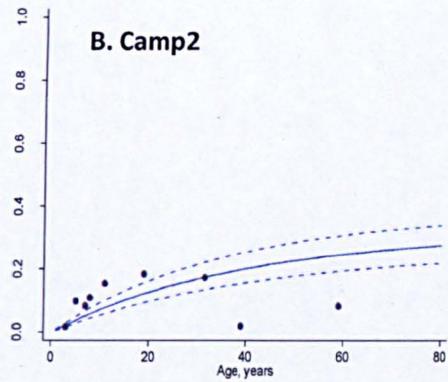
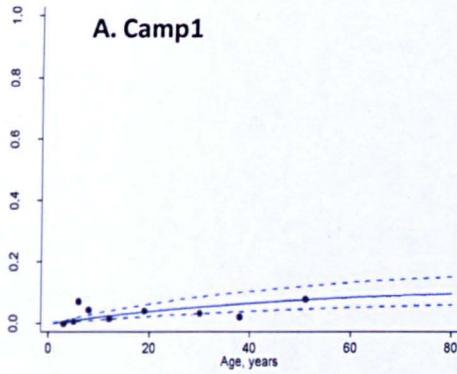
Filled circles represent observed data and the line is the fitted equation 1 in chapter 3 section 3.5.1.3. The proportion of seropositive individuals is represented by the vertical axis in each age group; the horizontal axis is the midpoint age of each age group, while dashed lines indicating 95% confidence intervals by likelihood ratio test.

Appendix 7PvAMA-1 age specific seroprevalence plots in each studied camp of KP A) camp1 B) camp2 C) camp3 D) camp4 E) camp5 F) all camps combined data.



Filled circles represent observed data and the line is the fitted equation 1 in chapter 3 section 3.5.1.3. The proportion of seropositive individuals is represented by the vertical axis in each age group; the horizontal axis is the midpoint age of each age group, while dashed lines indicating 95% confidence intervals by likelihood ratio test.

Appendix 8PvMSP-1₁₉ age specific seroprevalence plots in each studied camp of KP
 A) camp1 B) camp2 C) camp3 D) camp4 E) camp5 F) all camps combined data.



Filled circles represent observed data and the line is the fitted equation 1 in chapter 3 section 3.5.1.3. The proportion of seropositive individuals is represented by the vertical axis in each age group; the horizontal axis is the midpoint age of each age group, while dashed lines indicating 95% confidence intervals by likelihood ratio test.

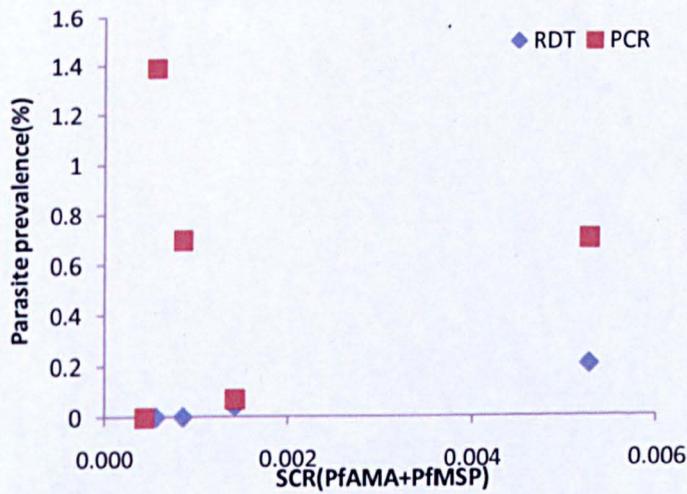
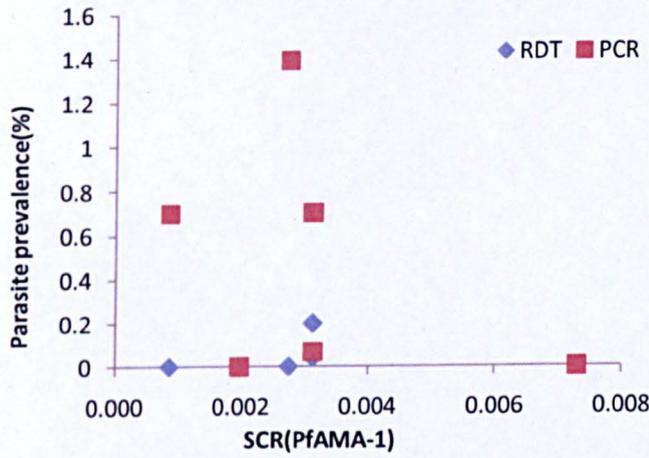
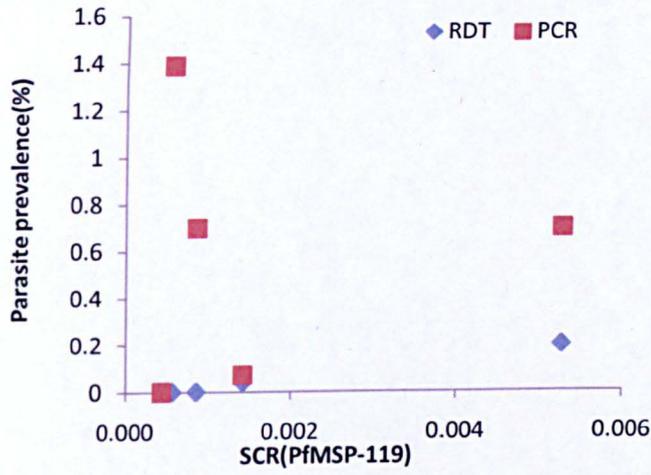
Appendix 9P. *falciparum*AMA, MSP and combined an gens seroconversion rates for each camp

Camp	PfAMA-1		PfMSP-1 ₁₉		Combined	
	SCR	CIs	SCR	CIs	SCR	CIs
1	0.002	(0.0011-0.0034)	0.0004	(0.0001-0.0014)	0.0024	(0.0015-0.0040)
2	0.0073	(0.0054-0.0098)	0.0004	(0.0001-0.0014)	0.0073	(0.0054-0.0098)
3	0.0028	(0.0017-0.0043)	0.0006	(0.0002-0.0015)	0.0034	(0.0022-0.0051)
4	0.0009	(0.0004-0.0019)	0.0009	(0.0004-0.0019)	0.0017	(0.0010-0.0031)
5	0.0031	(0.0020-0.0048)	0.0053	(0.0037-0.0075)	0.0078	(0.0059-0.0104)
All Camps	0.0031	(0.0026-0.0038)	0.0014	(0.0011-0.00190)	0.0045	(0.0038-0.0052)

Appendix 10 Seroconversion rates calculated for *P. vivax*AMA, MSP and combined an gens for each camp

Camp	PvAMA-1		PvMSP-1 ₁₉		Combined	
	SCR	CIs	SCR	CIs	SCR	CIs
1	0.0140	(0.0111-0.0176)	0.0024	(0.0015-0.0040)	0.0161	(0.0129-0.0200)
2	0.0308	(0.0258-0.0368)	0.0080	(0.0060-0.0106)	0.0362	(0.0305-0.0430)
3	0.0429	(0.0365-0.0504)	0.0262	(0.0219-0.0314)	0.0627	(0.0537-0.0732)
4	0.0256	(0.0212-0.0308)	0.0043	(0.0030-0.0063)	0.0293	(0.0245-0.0351)
5	0.0073	(0.0054-0.0098)	0.0101	(0.0078-0.0130)	0.0168	(0.0136-0.0207)
All Camps	0.0225	(0.0206-0.0245)	0.0096	(0.0085-0.0108)	0.0297	(0.0275-0.0322)

Appendix 11 Comparison of *P. falciparum* prevalence (measured by RDT and PCR) and SCR



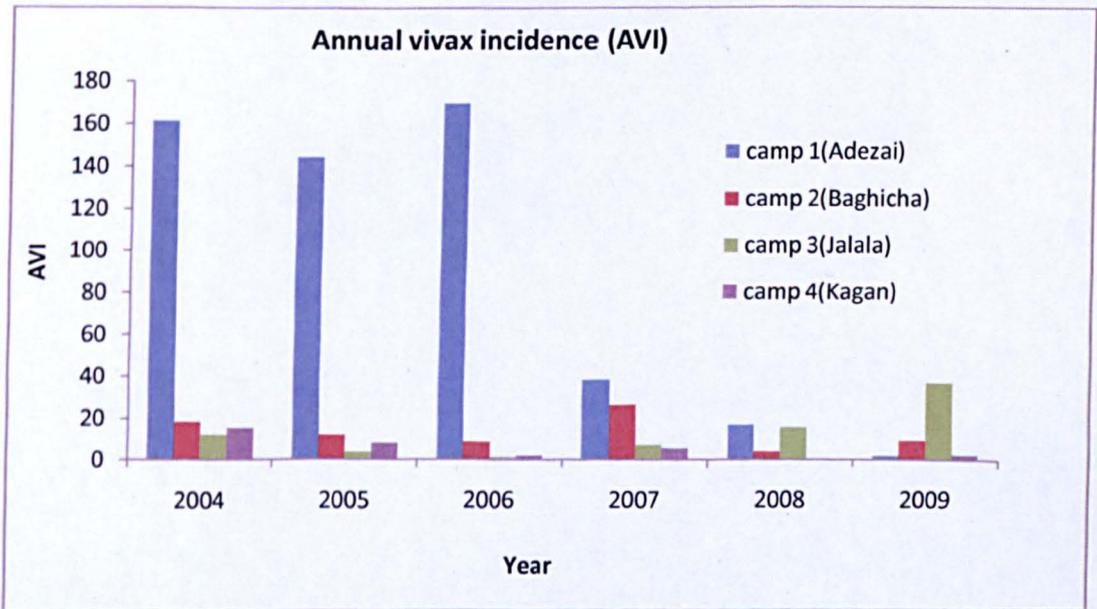
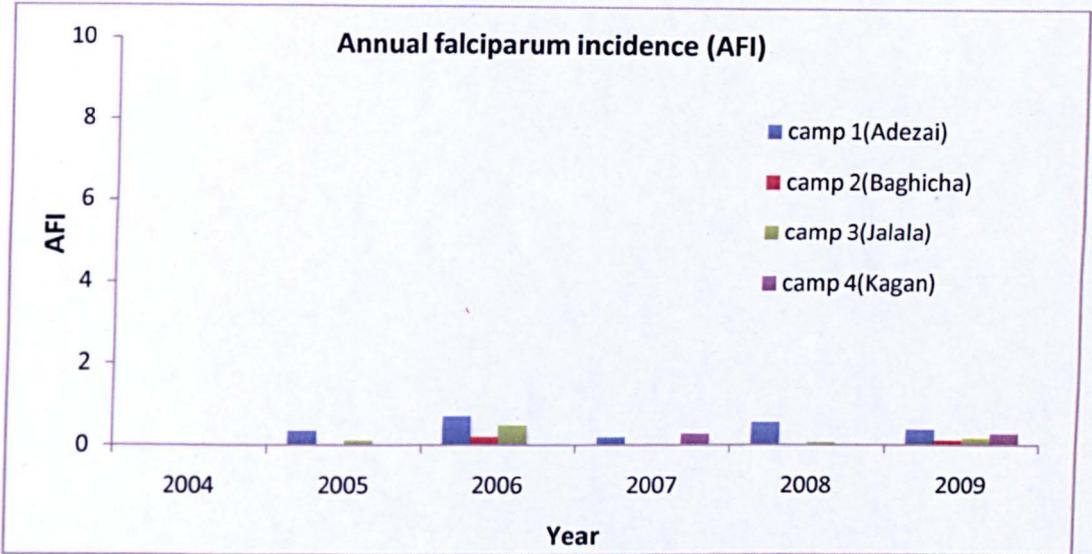
Appendix 12 Number and percentages of RDT positive and seropositive individuals against PvAMA-1 and PvMSP-1₁₉

<i>P. vivax</i>	PvMSP-1 ₁₉		PvAMA-1	
	-ve %(n)	+ve %(n)	-ve %(n)	+ve %(n)
RDT				
-ve	89.6(2169)	10.4(251)	77(1860)	23(556)
+ve	46.8(44)	53.9 (50)	64.6(62)	35.4(34)

Appendix 13 Number and percentages of PCR positive and seropositive individuals against PvAMA-1 and PvMSP-1₁₉

<i>P. vivax</i>	PvMSP-1 ₁₉		PvAMA-1	
	-ve %(n)	+ve %(n)	-ve %(n)	+ve %(n)
PCR				
-ve	84.5 (770)	15.5(141)	71.7 (652)	28.3(257)
+ve	60.1 (83)	39.9(55)	67.9(95)	32.2(45)

Appendix 14 Previous malaria data showing AVI and AFI trend in the studied camps recorded by BHUs of the studied camps from 2004-2005



AFI= Annual Falciparum Incidence/1000 Popula on

AVI= Annual Vivax Incidence/1000 Popula on

Appendix 15 Reagents used in Production of MSP119-GST using E.coli

Reagents

LB Broth

Added 25g of LB Broth powder to 1 litre deionised water, mixed, autoclaved, and stored at 4°C.

LB Agar

20g LB Agar powder was weighed transferred to a 500ml media bottle, which was filled up to 500ml mark with deionised water. The bottle was mixed well, autoclaved and stored at 4°C.

Terri c Broth (Sigma catalogue p.1530)

The following were weighed out and added to a 1 / autoclavable bottle:

Tryptone	12 g
Yeast extract	24 g
K ₂ HPO ₄	9.4 g
KH ₂ PO ₄	2.2 g

Suspended in 992 ml DI water and added 8 ml glycerol, mixed and autoclaved.

Ampicillin

2.50 g Ampicillin sodium was dissolved in deionised water (25 ml) to give a 100 mg/ml solution. Sterile filtered through a 0.22 µ filter and dispensed as 1 ml aliquots. Frozen at -20°C.

IPTG 100 mM

238 mg IPTG was dissolved in 10 ml deionised water. Sterile filtered through 0.22 µ filter and dispensed as 1 ml aliquots. Frozen at -20 °C.

Triton X-100 20% (v/v)

In a 50 ml conical-bottomed centrifuge tube 10 ml Triton X-100 was added to 40 ml water and mixed on roller mixer.

Glutathione Elution Buffer

10 mM reduced glutathione in 50 mM Tris-HCl (pH 8.0), was dispensed in 1-10 ml aliquots and stored at -20°C until needed.

LB-Amp plates

An aliquot of ampicillin from the freezer was removed and set out the appropriate number of Petri dishes. A 500 ml of LB-Agar was melted and allowed to cool until hand-hot. 0.25 ml of 100mg/ml ampicillin were added and mixed well. The plates were quickly poured, removing and replacing the dish lids so as to give the minimum chance of contamination. The plates were allowed to cool completely and when set were inverted, labeled and stored at 4°C.

LB-Amp medium

A tube of ampicillin (100 mg/ml) was thawed and 0.5 ml ampicillin was added aseptically to a 1 litre bottle of LB Broth, before mixing well by swirling.

Appendix 16 Reagents and preparation of buffers for ELISA

Reagents

- a) Controls (standard dilutions): Pooled positive serum from high transmission area in Tanzania
- b) SigmaFast OPD tablets (Sigma)
- c) Tween 20 (Sigma)
- d) Skimmed milk powder
- e) Horseradish peroxidase-conjugated rabbit anti-human IgG (Dako #P0214)
- f) NaH_2PO_4 (Sodium dihydrogen orthophosphate) VWR International Ltd
- g) Na_2HPO_4 (di-sodium hydrogen orthophosphate) VWR International Ltd
- h) NaCl (Sodium chloride) Fisher scientific
- i) H_2SO_4 (Sulphuric acid) BDH

Buffer solutions preparation

- a) Phosphate buffered saline (PBS) x10 pH 7.2

5.7g NaH_2PO_4

16.7g Na_2HPO_4

85g NaCl in 1litre distilled water at pH of 7.2

Stored at room temperature and disposed of after one month.

- b) Coating buffer pH 9.4-9.6

1.59g Na_2CO_3 and 2.93g NaHCO_3 was dissolve in 1litre distilled water at pH 9.5 and stored at 4°C, disposed of after one month.

- c) OPD substrate solution. A pair of Sigmafast OPD Buffer tablet was dissolved in 20ml distilled water and was used immediately and remaining solution was disposed of after use.

- d) PBS/Tween wash solution (PBST)

500ml 10x PBS was diluted with 4.5litre distilled H_2O , 2.5ml Tween (0.05% Tween in PBS) was added and the unused solution was disposed of at the end of each day.

- e) Blocking solution (1% skimmed milk powder in PBST)

10g of skimmed milk powder was mixed with 1litre PBST at ambient temperature

- f) 2M H_2SO_4

107 ml conc. H_2SO_4 was slowly added to 800ml distilled water.

Appendix 17 Computer-randomized list used for the selection of a household from a compound comprised of more than one households

Random numbers	Household head's Name
815	
238	
16	
458	
394	
437	
728	
576	
242	
780	

The names were recorded as given below and was written in the order they were given.

Random numbers	Name
815	Afzal
238	Ahmad
16	Bilal
458	Jaweed
378	Amir
437	
728	
576	
242	
780	

Next, household head with the lowest number were selected. If they refused, the next lowest number was selected.

Random numbers	Name
815	Afzal
238	Ahmad
16	Bilal
458	Jaweed
394	Amir
437	

Appendix 18 Computer-randomized list used for the selection of studied samples

<5 years		6-20 years		>20 years	
Random	Name	Random	Name	Random	Name
815		573		474	
238		21		343	
16		263		835	
458		4		72	
394		117		111	
437		81		795	
728		766		940	
576		903		763	
242		965		25	
780		935		241	

The head of the selected household was asked to give all the (first) names of the children under or equal to 5 years, then all the names of those 6-20 years and then all the names of those above 20 years. The names were recorded as given below and was written in the order they were given by the head of the family.

<5 years		6-20 years		>20 years	
Random	Name	Random	Name	Random	Name
815	Afshan	573	Leila	474	Osman
238	Belour	21	Fatima	343	Parween
16	Bilal	263	Ali	835	Fatima
458	Jaweed	4	Mitra	72	
378	Amir	117		111	
437		81		795	
728		766		940	
576		903		763	
242		965		25	
780		935		241	

Next, individuals with the lowest number were selected. If they refused, the next lowest number was selected.

<5 years	
Random	Name
	Afshan
815	
	Belour
238	
16	Bilal
	Jaweed
458	
	Amir
394	
437	

6-20 years	
Random	Name
	Leila
573	
	Fatima
21	
263	Ali
	Mitra
4	
117	
81	

>20 years	
Random	Name
	Osman
474	
	Parween
343	
835	Fatima
72	
111	
795	

Appendix 19 Peshawar university ethics committee approval

DEPARTMENT OF PHARMACY

University of Peshawar, N.W.F.P, Pakistan

Ref. 29/Pharm



Date: 26-05-2010

ETHICAL APPROVAL FORM

Application number 02/EC/Pharm
Date 20.05.2010
Name of Principal Investigator Ms. Sobia Wahid
Department Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK
Head of the Department Prof. Dr. Simon Croft
Name of Supervisor Dr. Chris Drakeley (Sr. Lecturer)
Name of Co-investigator Prof. Dr. Akram Shah
Department Of Zoology, University of Peshawar
Title: "Assessment of Exposure & Risk Factors for Malaria in Afghan Refugees NWFP (KPK) Pakistan".

* New Name of NWFP is Khyber Pakhtunkhwa

This application is approved by the committee

Prof. Dr. Fazal Subhan	(Convener)	
Prof. Dr. Zafar Iqbal	(Member)	
Prof. Dr. Zahoor Ahmad Swati	(Member)	
Prof. Dr. Akram Shah	(Member)	
Dr. Muhammad Ismail	(Member)	<u>Apologies</u>
Dr. Saeed-ur-Rahman	(Member)	<u>Apologies</u>
Dr. Attaullah	(Member)	<u>Apologies</u>
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Appendix 20 London School of Hygiene and Tropical Medicine ethics committee approval

LONDON SCHOOL OF HYGIENE
& TROPICAL MEDICINE

ETHICS COMMITTEE



APPROVAL FORM

Application number: 5715

Name of Principal Investigator **Sobia Wahid**

Department **Infectious and Tropical Diseases**

Head of Department **Professor Simon Croft**

Title: **Assessment of exposure and risk for malaria in Afghan refugee camps in North West Frontier Province (NWFP), Pakistan.**

This application is approved by the Committee.

Chair of the Ethics Committee

Date 24 June 2010

Approval is dependent on local ethical approval having been received.

Any subsequent changes to the application must be submitted to the Committee via an E2 amendment form.

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