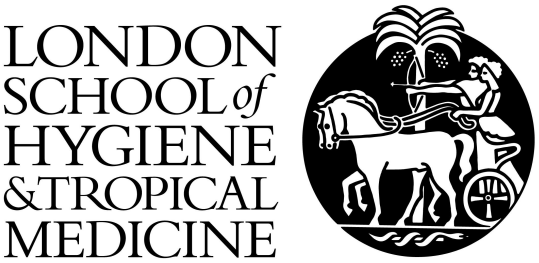

Epidemiology of malaria in the provinces of Sarangani, South Cotabato and Tawi-Tawi in Mindanao, The Philippines

MARY GRACE BINARAO DACUMA

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Department of Immunology & Infection
Faculty of Infectious & Tropical Diseases
London School of Hygiene & Tropical Medicine

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DECLARATION

I, Mary Grace Binarao Dacuma, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed Date

Mary Grace Binarao Dacuma 10 April 2015

ABSTRACT

The Philippines is targeting malaria elimination by 2020. To reach this goal, it is important to locate all residual foci of malaria and where possible, aggressively diagnose and treat every malaria infection. In low endemic provinces malaria transmission becomes focal in hard-to-reach areas where asymptomatic people do not actively seek treatment and thus, continue to perpetuate transmission. This study aimed (a) to estimate prevalence of *Plasmodium* species in three malaria-endemic provinces in Mindanao, (b) to measure malaria transmission intensity in these provinces using antibody markers of exposure to *P. falciparum* and *P. vivax* AMA-1 and MSP-1_{19} antigens, (c) to determine polymorphisms in *pfcrt, pfmdr1* and *pvmdr1* genes, and (d) to discuss implications of these findings to malaria elimination in Mindanao. Cross-sectional surveys were conducted to a total of 2,628 consenting participants across all ages in the provinces of Sarangani, South Cotabato and Tawi-Tawi from 2010 to 2013. The RDT FalciVax™ was used for field diagnosis of malaria Sarangani Province and South Cotabato Province for *P. falciparum* and *P. vivax* infection while microscopy was used in Tawi-Tawi Province for field diagnosis of malaria. Finger-prick blood spots on filter paper were collected from participants for PCR diagnosis, genotyping of *pfcrt*, *pfmdr1* and *pvmdr1* genes, and screening antibodies to *P. falciparum* and *P. vivax* AMA-1 and MSP-1_{19} antigens using indirect ELISA. Blood spots were also collected from patients presenting with malaria symptoms from selected municipalities of Sarangani Province and South Cotabato Province as a pilot survey. Overall malaria prevalence by PCR was 3.7% in Sarangani Province, 10% in South Cotabato Province and 4.2% in Tawi-Tawi Province. *P. falciparum* prevalence by PCR was higher than *P. vivax* prevalence in Sarangani Province and Tawi-Tawi Province but the opposite was found in South Cotabato Province. There was one imported case of *P. malariae* in South Cotabato and there were no *P. knowlesi* and *P. ovale* infections found in the three provinces surveyed. There were disagreements in diagnosing *P. falciparum* and *P. vivax* using antigen detection, microscopy and PCR and these were attributed to sampling low parasite-density infections from small volume of peripheral blood spotted on filter paper. The *pfcrt* codons 72-76 haplotypes CVMNK (27.4%), CVIET

(59.7%) and SVMNT (9.7%) were described in 62 *P. falciparum* isolates from Mindanao. The *pfcrt* mutant A144T and L160Y alleles were not found among *P. falciparum* isolates with *pfcrt* K76T mutant allele but lacked the *pfcrt* A220S mutation. The *pfmdr1* 86N-184F-1034S-1042N-1246D haplotype, which was repeatedly associated with higher parasite survival following artemether-lumefantrine treatment, was found in seven *P. falciparum* isolates from Mindanao. Genotyped *P. vivax* isolates from Mindanao have the wild type *pvmdr1* 91N allele, which corresponded to *pfmdr1* codon 86. The *pvmdr1* Y976F mutant allele, which has been reported in chloroquine-resistant *P. vivax* in other countries, was found in 55.6% (5/9) *P. vivax* isolates successfully genotyped in this codon while the *pvmdr1* 1076L wild-type allele was found in three *P. vivax* isolates successfully genotyped in this codon. Combined seroprevalence to *P. falciparum* and *P. vivax* AMA-1 and MSP-1_{19} antigens suggested that exposure to *P. falciparum* was higher than exposure to *P. vivax* in Sarangani Province and Tawi-Tawi Province. Overall seroprevalence to *P. falciparum* and *P. vivax* was 18.9% and 14.6% in Sarangani Province respectively. In Tawi-Tawi Province the overall seroprevalence to *P. falciparum* and *P. vivax* was 18.2% and 12.9% respectively. The opposite was observed in South Cotabato Province where overall seroprevalence to *P. falciparum* (3.4%) was lower than the overall seroprevalence to *P. vivax*. The seroconversion rates (λ) for *P. falciparum* and *P. vivax* malaria were estimated using simple reversible catalytic models. In Sarangani Province the SCR for *P. falciparum* (0.014, 95%CI 0.010-0.020) was lower than SCR for *P. vivax* (0.019, 95% CI 0.010-0.036). A model allowing two forces of infection was used to estimate SCR for *P. falciparum* in Tawi-Tawi. Results suggested that there was a change in *P. falciparum* transmission in Tawi-Tawi Province approximately 25 years before the survey was conducted. The estimated SCR for *P. falciparum* was 0.041 (95% CI 0.017-0.098) in Tawi-Tawi Province before 1987. The model suggested that SCR was reduced to 0.007 (95% CI 0.005-0.009) after 1987 to the time of survey. In South Cotabato the SCR for *P. falciparum* was very low (0.004, 95% CI 0.001-0.016). There was no SCR estimated for *P. vivax* in South Cotabato because seropositivity was equally distributed across age groups. Findings in this study were held back by sample size and low-density parasite infections in small number of infected humans. Nevertheless, this provided important baseline data for malaria epidemiology in MIndanao.

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Mary Grace Dacuma

This is for Mamang, Papang and Daniel

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ACT	artemisinin-based combination therapy
AHA	acute haemolytic anaemia
AMA	apical membrane antigen
API	annual parasite incidence
AQ	amodiaquine
ARMM	Autonomous Region in Muslim Mindanao
AS	artesunate
ASTMH	American Society of Tropical Medicine and Hygiene
BC	before the Christian era
BLAST	Basic Local Alignment Search Tool
CDC	Center for Disease Control
CI	confidence interval
CQR	chloroquine resistance
DDT	dichlorodiphenyltrichloroethane
DHA	dihydroartemisinin
DHFR	dihydrofolate reductase
DHPS	dihydropteroate synthase
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DOH	Department of Health
DVS	dominant vector species
EIR	entomological inoculation rate
ELISA	enzyme-linked immunosorbent assay
GIDA	geographically isolated and disadvantaged area
G6PD	glucose-6-phosphate dehydrogenase
GMEP	Global Malaria Eradication Program
GPARC	Global Plan for Artemisinin Resistance Containment
IQR	interquartile range

IRS	indoor residual spraying
ITN	insecticide treated net
HRP2	histidine-rich protein 2
LGC	Local Government Code
LGU	local government unit
LLIN	long-lasting insecticide treated net
LSHTM	London School of Hygiene & Tropical Medicine
MCP	Malaria Control Program
MILF	Moro Islamic Liberation Front
MNLF	Moro National Liberation Front
MQ	mefloquine
MRL	Malaria Reference Laboratory
MSP	merozoite surface protein
MTI	malaria transmission intensity
NCBI	National Center for Biotechnology Information
NPV	negative predictive value
NTC	no template control
OD	optical density
OR	odds ratio
PAGASA	Philippine Atmospheric, Geophysical and Astronomical Services Administration
PBS	phosphate buffered saline
PCHRD	Philippine Council for Health Research and Development
PCR	polymerase chain reaction
Pf	*Plasmodium falciparum*
pfcrt	*P. falciparum* chloroquine resistance transporter gene
pfmdr1	*P. falciparum* multidrug resistance 1 gene
PHC	primary health care
pLDH	parasite-specific lactate dehydrogenase
PPV	positive predictive value
Pv	*Plasmodium vivax*
pvmdr1	*P. vivax* multidrug resistance 1 gene

PPQ	piperaquine
RBC	red blood cell
RBM	Roll Back Malaria
R_c	controlled reproduction number
RDT	rapid diagnostic test
RHU	rural health unit
R_o	basic reproduction rate
ROS	reactive oxygen species
SCR	seroconversion rate
SERCA	sarcoplasmic reticulum calcium-dependent ATPase
SNP	single nucleotide polymorphism
SP	sulfadoxine-pyrimethamine
SPR	slide positivity rate
TBE	Tris-Borate-EDTA buffer
TMB	tetramethylbenzidine
TMD	transmembrane domain
UK	United Kingdom
UNICEF	United Nations Children's Fund
UPLB	University of the Philippines Los Baños
USAID	United States Agency for International Development
WHO	World Health Organization
WWII	World War II

CHAPTER 1: Introduction

1.1. Malaria through time

Malaria in ancient times

Malaria is a recognised disease for centuries. The earliest likely reference on malaria was found in the Chinese medical classic, the Nei Ching, edited by Emperor Huang Ti in 2700 BC (Bruce-Chwatt, 1988). The ancient royal library of Ashurbanipal contained clay tablets recording deadly periodic fevers, most probably malaria in Mesopotamia in 2000 BC (Sherman, 1988). Even the Ebers Papyrus of Egypt in 1570 BC mentioned fever, spleen enlargement and possible curative potions for such illnesses. During the Indian Vedic period (1500 to 800 BC) autumnal fevers were referred to as the "king of diseases" (Bruce-Chwatt, 1988, Sherman, 1988). Whether all seasonal fevers and spleen enlargements in prehistoric times were all related to malaria remained uncertain as people in ancient times attributed sicknesses as retributions from displeased gods or workings of evil spirits (Gilles, 2002). It was only in the fifth century BC that the Greek physician Hippocrates (460-370 BC) dismissed superstition about causes of fever and suggested plausible reasons for periodic fever that occurred at a particular season of the year or among people residing near marshes (Gilles, 2002). For more than 2500 years the belief persisted that miasma rising from marshes caused seasonal fevers (Bruce-Chwatt, 1981, Cox, 2010).

Finding cure before cause of malaria

During the late 16th century Jesuit missionaries learned from Peruvian Indians about a bark's extract for treating swamp fever and brought this knowledge back to England (Butler et al., 2010). There was very sparse evidence that Peruvian Indians possessed this knowledge. According to the Spanish scholar Francisco Guerra the natives actually used the bark's extract to suppress shivering when they were exposed to damp and cold weather (Greenwood, 1992). In the 1630s the Spaniards discovered a different tree named *álbor de calenturas* or fever tree (Keeble, 1997), which in 1742 was named

Cinchona[1] by the Swedish botanist Linnaeus based from Charles Marie la Condamine's (1701-1774) descriptions while studying the tree in Loja[2] (Lee, 2002, Rodriguez, 2007). The Jesuits brought *Cinchona* to England for treatment of ague[3] but the protestant Church of England viewed this with distrust. Its use became extensive in England in 1671 when Sir Robert Talbor produced an effective remedy for ague using *Cinchona* extract as the secret ingredient. This brought him wealth and fame across Europe (Keeble, 1997). Quinine, the active ingredient of *Cinchona*, was finally isolated in 1820 (Ball, 2008).

Discovery of the malaria parasite

Swamp fevers are due to a germ.
-Charles Louis Alphonse Laveran (1845-1922)

In 1740 ague acquired the Italian term *mal'aria* from Horace Walpole's letter recounting the deadly fever visiting Rome every summer. It was the prominent British geologist John Macculloch who first used the word malaria in English scientific publication in 1827, which referred to the paludial origin of malaria (Bruce-Chwatt, 1977). The miasma theory weakened in the late 19th century upon Pasteur and Koch's discovery that microorganisms cause diseases. In 1879 Edwin Klebs and Corrado Tommasi-Crudeli reported that the microorganism *Bacillus malariae,* which they isolated from the air and mud in the Roman Campana was the cause of malaria (Smith and Sanford, 1985). However, Charles Louis Alphonse Laveran challenged their findings with the hypothesis that malaria was actually caused by a parasite of the red blood cells, which he observed upon examining the blood of people with malaria (Figure 1.1) (Smith and Sanford, 1985, Bruce-Chwatt, 1981). Laveran based his treatise on previous

[1] Linnaeus was inspired by the work of Sebastiano Bado recounting the romantic tale of the Countess of Chinchón, wife of Spanish viceroy of Peru in the early 17th century, who fell ill but was healed by the bark's curative powers. While the accounts of the Countess and her illness were proven fictitious, the tree's name persisted to this date as *Cinchona* BUTLER, A. R., KHAN, S. & FERGUSON, E. 2010. A brief history of malaria chemotherapy. *J R Coll Physicians Edinb,* 40**,** 172-7, RODRIGUEZ, F. M. 2007. Precisions on the history of quinine. *Reumatologia Clinica,* 3**,** 194-196.

[2] *Loja* is known in the present day as Ecuador.
[3] *Ague* was the English term for swamp fever before the Italian term mal'aria was coined.

works of Johann Heinrich Meckel (1847), and Virchow and Friedrich (1848) about the brown pigments they have observed in organs of people who died of intermittent fever (Bruce-Chwatt, 1981). However, from 1880 the scientific community favoring the bacterial cause of malaria was not receptive and was skeptical of Laveran's discovery[4] that it was only in 1890 before the malaria parasite gained general acceptance (Smith and Sanford, 1985).

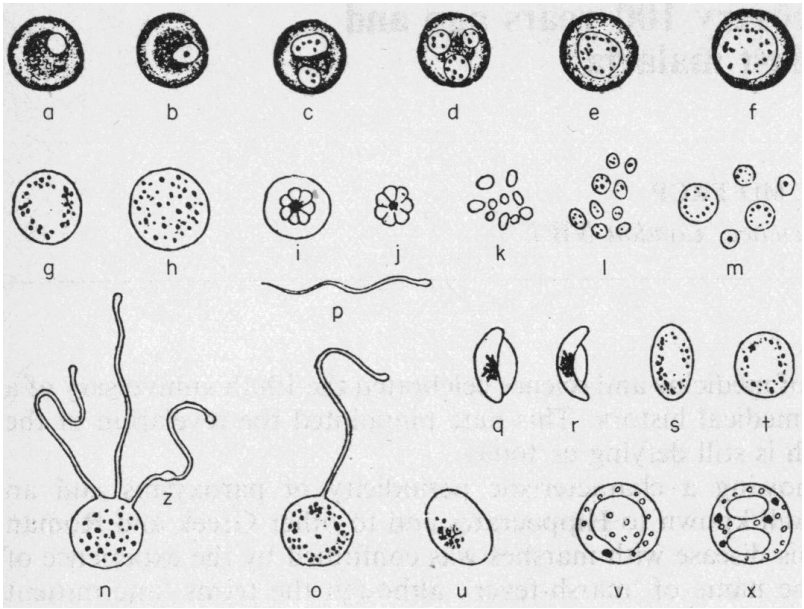


Figure 1.1. Malaria parasites observed, illustrated and named *Haemamoeba malariae* by Charles Louis Alphonse Laveran; published in *Comptes Rendus de l' Academie des Sciences,* 24 October 1881 and obtained from Sergeant & Sergent 1929 (Bruce-Chwatt, 1981).

Laveran named the malaria parasite *Oscillaria malariae*, which was later changed to *Haemamoeba* and eventually to *Plasmodium.* The name *Plasmodium,* which was originally used for a vegetative slime fungus, was used for malaria parasites from 1885

[4] Smith and Sanford (1885) discussed in length several oppositions to Laveran's malaria parasite in blood. SMITH, D. C. & SANFORD, L. B. 1985. Laveran's germ: the reception and use of a medical discovery. *Am J Trop Med Hyg,* 34**,** 2-20.

onwards (Bruce-Chwatt, 1988). Six years after finding the first *Plasmodium* in blood, Camillo Golgi discovered that *P. vivax* and *P. malariae* were responsible for tertian and quartan fevers, respectively (Sherman, 1988). In 1892 Marchiafava and Bignami found the cause of aestivo-autumnal fever and described *P. falciparum* for the first time (Gilles, 2002).

Recognising the vector of malaria

Despite Laveran's discovery, the means of natural transmission of malaria from person to person remained unknown. This mystery was solved in 1897 when the British Surgeon-Major Ronald Ross observed pigmented cells in the wall of the stomach of *Anopheles* mosquito fed with blood from a person with malaria (Ross, 1897, Ross, 1898). This elucidated the role of mosquitoes in malaria transmission. However, speculations on the role of mosquitoes to malaria transmission have already been entertained prior to Ross's singular discovery. In 1717 the greatest Italian physicist of his time Giovanni Maria Lancisi wrote a treatise that marsh insects probably ingested miasma and injected this into human blood (Russell, 1943). In 1883 Albert Freeman Africanus King made the closest incrimination to date of the mosquito in malaria transmission (Russell, 1943). Following Ross's discovery Battista Grassi (1854-1925) and colleagues began working on the life cycle of the malaria parasites (Cook, 1997). Their experiments were repeated by Manson and colleagues in London and in the Roman Campana, which finally shed understanding how mosquitoes transmit malaria from person to person in 1900 (Manson, 2002). The vital role of the female *Anopheles* to the life cycle of *Plasmodium* will be discussed in depth in later sections describing the life cycle of the *Plasmodium* parasites.

1.2. The *Plasmodium* parasites, public health burden & vectors

The hundred years following the discovery of the malaria parasite in blood and its mode of transmission from person to person have seen advancements in the *Plasmodium* biology, control and treatment (Najera, 1999a, Gardner et al., 2002, Holt

et al., 2002, McCutchan et al., 1984). To date malaria is still an important global health problem with 3.4 billion people at risk of acquiring the infection (World Health Organization, 2013). Malaria is caused by any of the six *Plasmodium* species capable of infecting humans - *P. falciparum, P. vivax, P. malariae* (Mendis et al., 2001), non-recombining sympatric species *P. ovale wallikeri* and *P. ovale curtisi* (Sutherland et al., 2010), and the zoonotic *P. knowlesi* (Singh et al., 2004). These species can be transmitted from person to person through the bite of an infected female *Anopheles* mosquito (Jeffery, 1960, Young and Burgess, 1961, Manson, 2002), except *P. knowlesi* wherein known transmission is only from macaque to humans (Vythilingam et al., 2006). Malaria can be asymptomatic, uncomplicated or severe (Boyd and Kitchen, 1937, Adeloye et al., 1971, Allen et al., 1996) depending upon several factors such as infecting *Plasmodium* species, erythrocyte invasion, multiplication and survival inside the host, and host immunity (Miller et al., 1994, Wipasa et al., 2010, Polley et al., 2006).

P. falciparum (Welch)

Among the species of malaria parasites capable of infecting humans, *P. falciparum* is the deadliest (Triglia et al., 2001), particularly among children under five years (Allen et al., 1996). It can invade erythrocytes at all stages of development with multiple invasion of a single erythrocytes (Wilson et al., 1977, Simpson et al., 1999), causes sequestration of infected erythrocyte in microvasculature of organs and initiates a cascade of cytokine-driven pathology (David et al., 1983, Ho et al., 1991, Ringwald et al., 1993). Aside from these, *P. falciparum* has already developed resistance to most antimalarials (Payne, 1987, Roper et al., 2004, Dondorp et al., 2009). In 2010 about 2.5 billion people were at risk of *P. falciparum* infection of which 31% were in Africa, 65% were in Central and South East Asia, and 3.5% in America (Figure 1.2) (Gething et al., 2011).

P. vivax (Grassi and Feletti)

The severity of *P. falciparum* malaria overshadows the huge burden of *P. vivax*, which has the widest geographic spread outside of Africa (Mendis et al., 2001, Gething et al., 2012). Of the 2.48 billion people at risk of *P. vivax* infection in 2010, 91% were in Central and South East Asia, 6% in the Americas and only 3% in Africa (Figure 1.3) (Gething et al., 2012). Unlike *P. falciparum, P. vivax* only infects reticulocytes and requires the Duffy antigen for erythrocyte invasion (Chitnis et al., 1996). *P. vivax* can cause severe malaria (Tjitra et al., 2008, Barcus et al., 2007, Song et al., 2007, Kochar et al., 2009) and in the recent years *P. vivax* has developed resistance to chloroquine (Rieckmann et al., 1989) and antifolates (Lu et al., 2010). It has developed alternative pathway to infect Duffy negative individuals (Menard et al., 2010, Mendes et al., 2011). Lastly, its capacity to form hypnozoites makes it difficult to control and eliminate (Krotoski et al., 1982).

P. ovale (Stephens) and *P. malariae* (Marchiafava and Bignami)

P. ovale is a malaria parasite capable of infecting humans and primates (Jeffery et al., 1954, Bray, 1957, Kaiser et al., 2010). It is widely distributed in Africa and the Asia-Pacific as two distinct non-recombining sympatric species *P. ovale curtisi* (classic form) and *P. ovale wallikeri* (variant form) (Lysenko and Beljaev, 1969, Sutherland et al., 2010, Oguike et al., 2011). *P. ovale* spp. can cause relapses because of its ability to form liver stage hypnozoites as a significant part of its life cycle (Garnham et al., 1955, Dixtt, 1958). Infections with *P. ovale* spp. are commonly diagnosed in children but these can also persist as low frequency infections among adults (Faye et al., 2002, Dinko et al., 2013). Similar to *P. ovale*, *P. malariae* is a malaria parasite that infects primates and humans (Rodhain, 1948). *P. malariae* can cause relapse after years of asymptomatic infection (Bray, 1959, Chadee et al., 2000, Morovic et al., 2003). Both *P. ovale* and *P. malariae* occur in low frequency compared to *P. falciparum* and *P. vivax* but high prevalence of *P. ovale* and *P. malariae* has already been demonstrated in some regions (Zhou et al., 1998, Doderer-Lang et al., 2014).

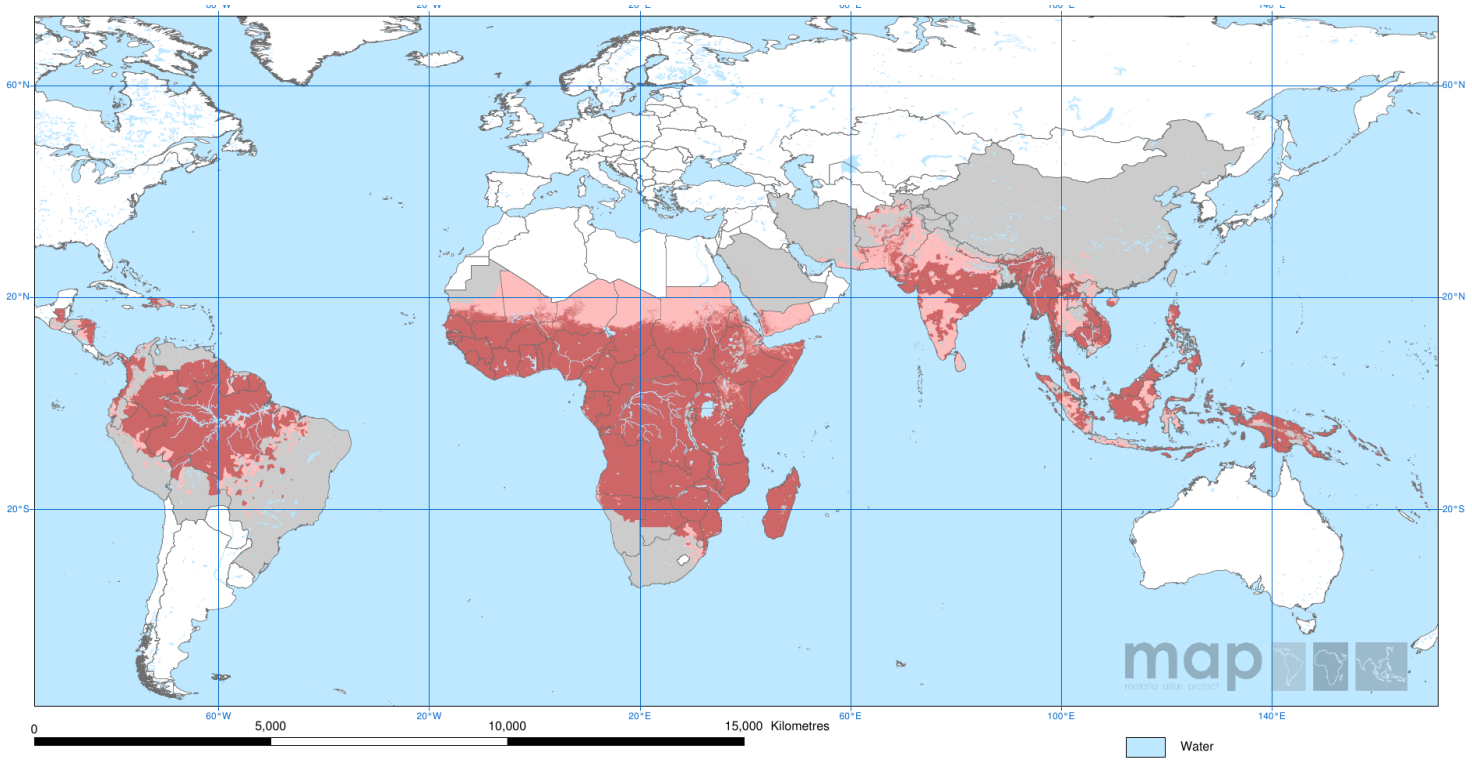


Figure 1.2. Spatial limits of *P. falciparum* transmission in 2010. Grey areas are *P. falciparum*-free; pink areas have unstable *P. falciparum* transmission (API<0.1 per 1,000 people per annum); red areas have stable *P. falciparum* transmission (API>0.1 per 1,000 people per annum) (Gething et al., 2011).

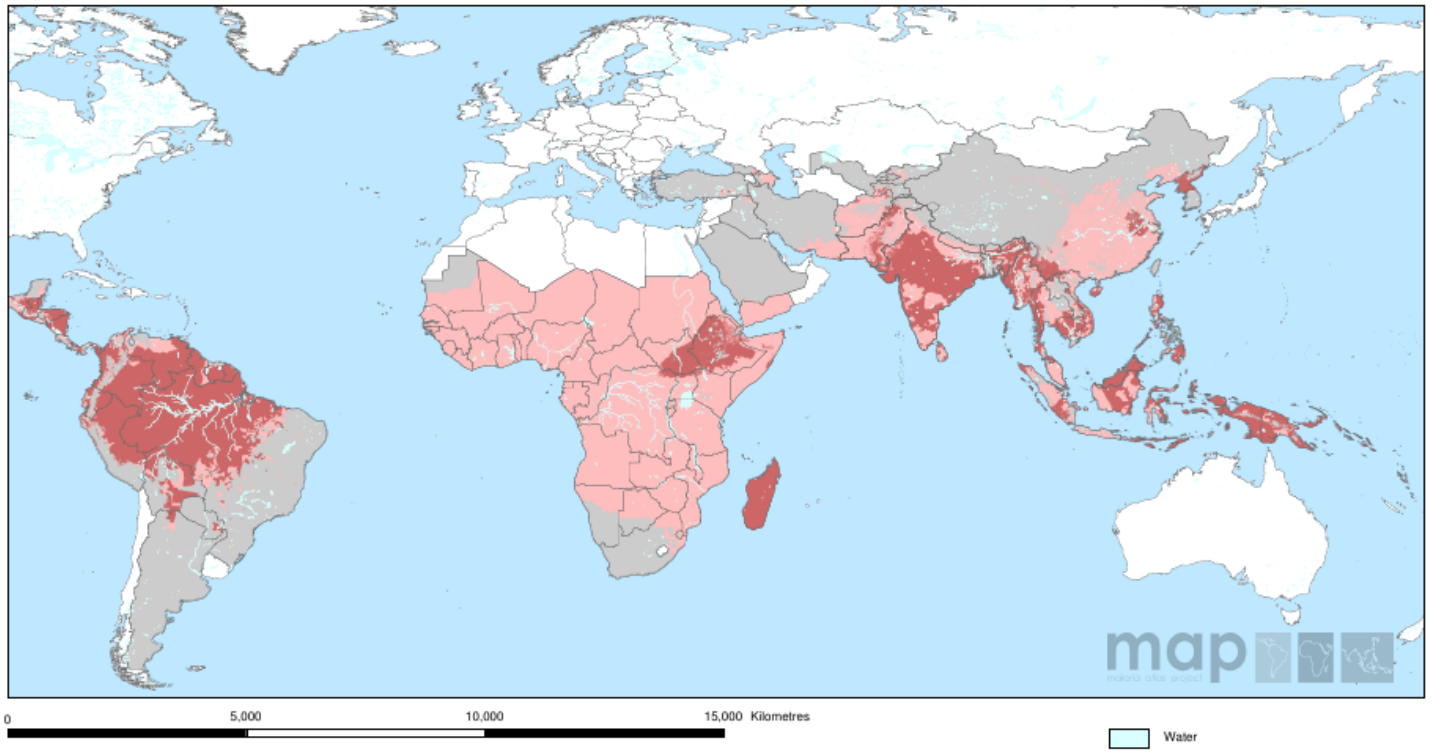


Figure 1.3. Spatial limits of *P. vivax* transmission in 2010. Grey areas are *P. vivax*-free; pink areas have unstable transmission of *P. vivax* (API<0.1 per 1,000 people per annum); red areas have stable *P. vivax* transmission (API>0.1 per 1,000 people per annum) (Gething et al., 2012).

P. knowlesi (Sinton and Mulligan)

P. knowlesi is a malaria parasite naturally infecting Old World monkeys and is capable of infecting humans (Garnham, 1966, Chin et al., 1968). This was first experimentally transmitted to humans in 1932 and the first natural human infection was reported in 1965 (Chin et al.). *P. knowlesi* has unique 24-hour schizogonic cycle, with potential to cause severe malaria in humans (Galinski and Barnwell, 2009, Cox-Singh et al., 2010). This parasite has gained recent attention because of natural human infections reported in Malaysia (Singh et al., 2004), Thailand (Jongwutiwes et al., 2004, Sermwittayawong et al., 2012), Philippines (Luchavez et al., 2008), Singapore(Ng et al., 2008) and Viet Nam (Van den Eede et al., 2009) among others. Natural human infections with *P. knowlesi* have been present for years and thus, this zoonotic malaria is not recently emergent (Lee et al., 2009). *P. knowlesi* has the propensity to be misdiagnosed using microscopy because its ring and trophozoite band stages resemble that of *P. falciparum* and *P. malariae* respectively (Singh et al., 2004). To date, there is limited information on the extent of *P. knowlesi*'s threat to public health (Sullivan, 2010) and there are no reports yet of its natural human-to-human transmission.

Anopheles vectors

Ronald Ross's discovery of *Plasmodium* in the stomach wall of female *Anopheles* mosquito fed with infected human blood elucidated its role in malaria transmission (Ross, 1897, Manson, 2002). Since then it has been established that contact between *v*ector and host is important in perpetuation of malaria (Bruce-Chwatt et al., 1966, Dye and Hasibeder, 1986). The host-seeking behavior of female *Anopheles* as well as its biting time and resting period after blood feeding vary per species (Githeko et al., 1996, Reddy et al., 2011, Killeen et al., 2001). Anthropophilic species like *An. gambiae* and *An. funestus* that prefer humans are considered efficient vectors of malaria compared to zoophagic species like *An. arabiensis* that prefer animals (Bockarie et al., 1996, Githeko et al., 1994). Parous *Anopheles* species with sporozoites of *P. falciparum* bite late at night than their nulliferous forms (Bockarie et al., 1996).

To date there are 462 *Anopheles* species formally named and 70 have been identified as efficient vectors of malaria (Harbach, 2004, Hay et al., 2010). In an endemic area majority of malaria are spread by dominant vector species (DVS) or species complex of *Anopheles* because of their preference for humans, abundance and their adult stage live long enough to transmit the parasite (Hay et al., 2010). Species complex refers to closely related *Anopheles* species, which are phenotypically indistinguishable, may be sympatric but still showing behavioral differences (Sinka et al., 2011).

There are seven *Anopheles* dominant vector species or species complex that transmit malaria in Africa. These include *Anopheles arabiensis, An. funestus, An. gambiae*, *An. melas, An. merus, An. moucheti* and An*. nili* (Figure 1.4) (Sinka et al., 2010) among which *An. gambiae* remains the most efficient vector in sub-Saharan Africa (Coetzee, 2004). This species is anthropophilic, rests indoors, long-lived with short larval period and often found in areas of human habitation (Githeko et al., 1994, Githeko et al., 1996, Sinka et al., 2010). In Europe and the Middle Eastern Region the six DVS of malaria include *Anopheles atroparvus, An. labranchiae, An. messeae, An. sacharovi, An. sergentii* and *An. superpictus* (Figure 1.4) (Sinka et al., 2010). There are 19 DVS in the Asia-Pacific Region, which include *An. aconitus, An. annularis, An. balabacensis, An. barbirostris, An. culicifacies, An. dirus, An. farauti, An. flavirostris, An. koliensis, An. lesteri, An. leucosphyrus* and *An. latens, An. maculatus* group, *An. punctulatus, An. sinensis, An. subpictus,* and *An. sundaicus* (Harbach, 2004, Sinka et al., 2011). An*. hackeri* was the first identified natural vector of *P. knowlesi* along the Malayan coast (Wharton and Eyles, 1961), *An. dirus* was identified as *P. knowlesi* vector in Southern Vietnam (Marchand et al., 2011) while *An. latens* has been implicated in transmitting *P. knowlesi* from monkeys to humans in Peninsular Malaysia (Vythilingam et al., 2006). Taking into account the behavior of these DVS and species complexes can impact the success in malaria control of a region.

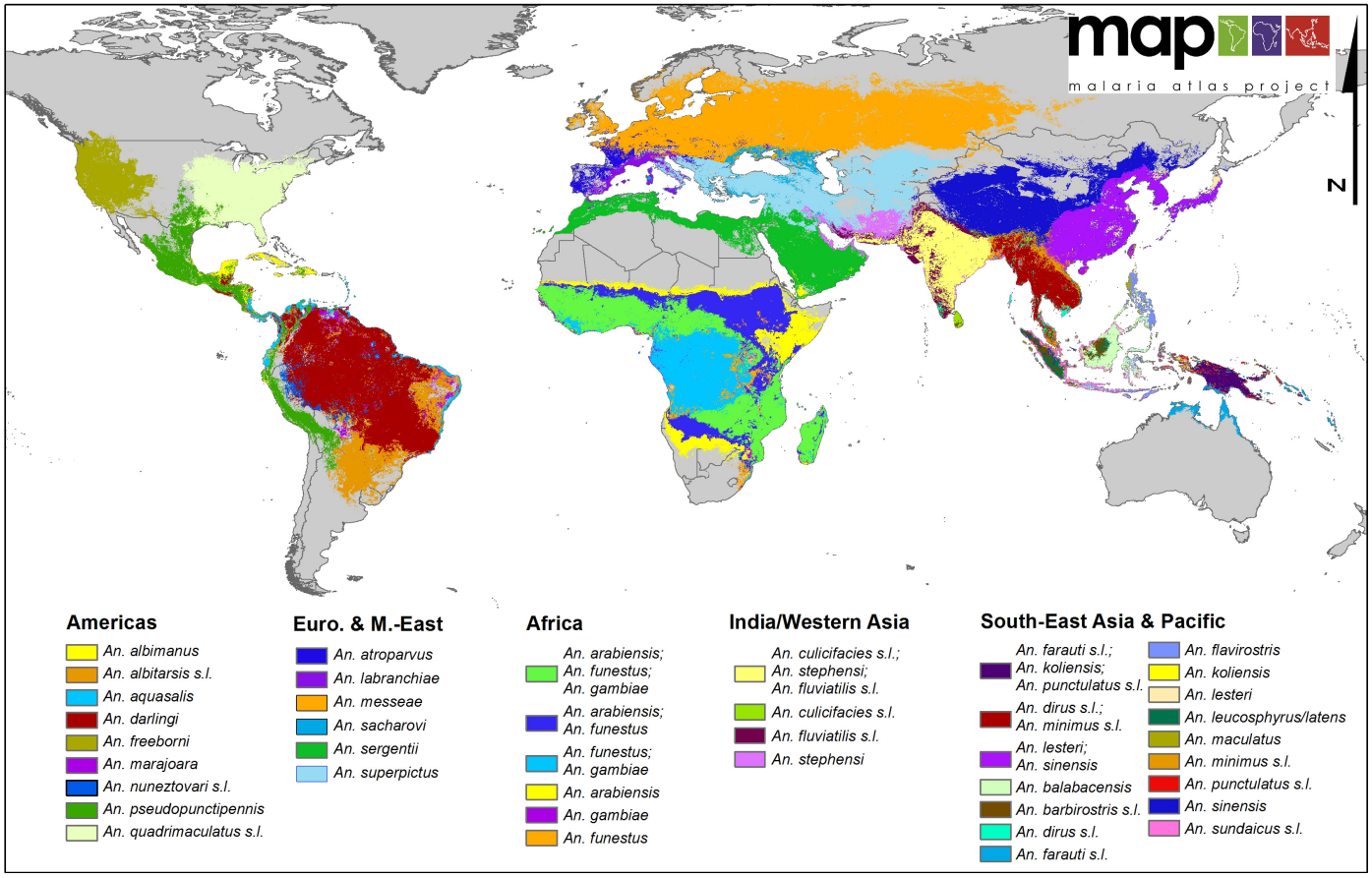


Figure 1.4. Distribution of dominant malaria vectors across the world (Sinka et al., 2012).

1.3. The *Plasmodium* life cycle

The *Plasmodium* parasites share a common life cycle with minor variations depending upon the species (Figure 1.5). This complex life cycle is key to understanding malaria transmission and pathogenesis in developing strategic measures for malaria treatment, control and elimination (Alonso et al., 2011, Sinden et al., 2012).

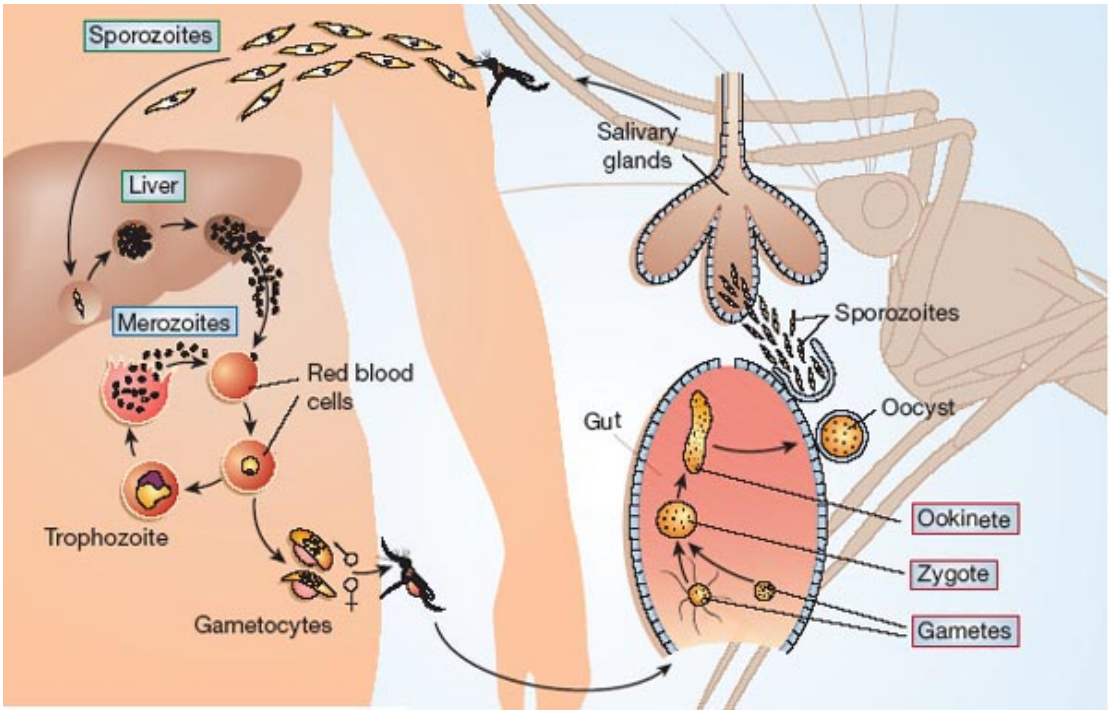


Figure 1.5. Life cycle of *Plasmodium* in the mosquito vector (sexual stages) and the human host (asexual stages) (Menard, 2005).

Inside the vertebrate host

The works of Manson and Ross incriminated the role of *Anopheles* in the transmission of malaria parasites through studies on bird malaria (Ross, 1897, Ross, 1898). Sporozoite, which is the infective stage of malaria parasites, would be injected into the

next unsuspecting host through the bite of a mosquito (Shortt, 1951c). Inside the vertebrate host, Grassi (1900) initially proposed that sporozoites developed in tissues before its asexual form would infect erythrocytes but aborted this in favour of Schaudinn's (1902) claim that sporozoites directly penetrated erythrocytes (Shortt, 1951a). However, Schaudinn's theory was discredited when James and Tate (1937) demonstrated in bird malaria that sporozoites invaded other tissues before infecting erythrocytes and referred to this as the exoerythrocytic schizogony (James and Tate, 1938). The search for the organ where sporozoites would undergo exoerythrocytic schizogony in mammals took several years before Shortt and Garnham (1948) provided the strongest proof demonstrating exoerythrocytic stages of *Plasmodium* in the liver. A full historical account leading to this discovery was given by Shortt (1951a).

Inside the liver cell, sporozoites round up and undergo mitotic division to form multinucleated schizont that later on ruptures to release single nucleated merozoites, which will either infect erythrocytes or other liver cells to cause relapses in some forms of malaria parasites (Shortt and Garnham, 1948, Shortt, 1951c, Shortt et al., 1948). In *P. ovale* and *P. vivax*, a proportion of liver-stage parasites remain dormant as hypnozoite (Wiseman, 1970, Chin and Coatney, 1971, Krotoski et al., 1982). Among non-relapsing malaria parasite species, *P. malariae* has prolonged period of latency following primary infection (Geiger and Kelly, 1916) and for several years this has been attributed to persistent low density infection in the blood (Corradetti, 1966, Cogswell, 1992). There are clinical data suggesting that *P. malariae* produces hypnozoites in the liver as part of its life cycle (Sutherland et al. unpublished data). To date, *P. malariae* hypnozoite has not been demonstrated yet in the liver.

From infected liver cells merozoites are released in vesicles called merosomes, which protect merozoites from being attacked by the immune system (Sturm et al., 2006). Merozoites invade erythrocytes in a complex process involving interactions between erythrocyte-binding ligands of merozoites with surface receptors on the erythrocytes (Dvorak et al., 1975, Sam-Yellowe et al., 1988, Rayner et al., 2000). Inside erythrocytes merozoites transform into ring or trophozoite stage, which is metabolically active and utilise haemoglobin as source of amino acids for growth and asexual reproduction

(Goldberg et al., 1990). At the end of the trophic stage mature trophozoites undergo erythrocytic schizogony, which produces merozoites that are released upon rupture of infected erythrocyte to infect other erythrocytes (Shortt, 1951c). After several erythrocytic cycles, some merozoites differentiate into male and female gametocytes (Rowley-Lawson, 1911, Shortt, 1951c) and this sexual differentiation is regulated by genetic (Dechering et al., 1997) and environmental factors (Carter and Miller, 1979, Bruce et al., 1990, Williams, 1999).

Inside the *Anopheles* vector

Gametocytes are released from erythrocytes in the mosquito midgut following ingestion of blood from a malaria-infected person. Exflagellation is initiated by rise in blood pH (Carter and Nijhout, 1977), some factors found in the mosquito midgut (Nijhout, 1979, Martin et al., 1978), and a reduction in temperature (Billker et al., 1997) among others. During exflagellation the male gametocyte undergoes meiotic division of the nucleus producing up to eight nuclei and forms cytoplasmic processes or flagella that fill up with nuclear material before detaching as highly motile microgametes (Shortt, 1951c). The female gametocytes or macrogametocytes also undergo meiotic division to form haploid macrogamete, which will be fertilised by the microgamete to form the zygote. Formation of the zygote establishes malaria infection to the vector (Dearsly et al., 1990). Within 24 hours the zygote transforms into a motile ookinete, which crosses the midgut epithelium and become an oocyst (Kumar et al., 1985). The oocysts undergo sporogony to form haploid sporozoites that bud from the oocysts and migrate into the salivary glands of the mosquito for transmission (King, 1916, Shortt, 1951c, Shute and Maryon, 1952).

1.4. Global malaria control to eradication

Following the discovery of the vital role of *Anopheles* in malaria transmission, malaria control in the early 1900s focused on annihilating mosquito vectors with remarkable success in the Panama Canal and complete eradication of introduced *An. gambiae* in Brazil (Gubler, 1998, Russell, 1943). Dusting with larvicide Paris green was introduced in the 1920s (The Rockefeller Foundation, 1926) and gained popularity in controlling malaria until the 1930s. In 1939 the discovery of the long-lasting insecticidal properties of dichlorodiphenyltrichloroethane (DDT) modernised indoor residual spraying (IRS) and this led to dramatic reduction in malaria transmission (World Health Organization, 2011a). The synthetic antimalarial chloroquine, which was found to be effective and safe, was made available in the 1940s as part of the World War II (WWII) United States Army drug development programme (World Health Organization, 2011a). With the availability of DDT and chloroquine, confidence was renewed that malaria could be eliminated. However, in 1953 DDT resistance was first reported in Greece (Livadas and Georgopoulos, 1953). This event was actually the prime mover of urgent planning for malaria eradication so DDT use would be terminated at the soonest possible time before the anticipated development of widespread *Anopheles* resistance (Payne, 1987, Bruce-Chwatt, 1988).

In 1955 the World Health Organization (WHO) launched the "Global Malaria Eradication Programme" (World Health Organization, 1957) with time-limited phases designed in 1956 (Figure 1.6) that recognised the necessity to eradicate malaria before widespread insecticide resistance (Najera et al., 2011) . The first year of the campaign was the preparatory phase, followed by three to four years attack phase using residual spraying to interrupt and reduce transmission, and thereafter a consolidation phase when remaining foci would be removed by antimalarial drugs (Bruce-Chwatt, 1988). Unlike malaria control that aims to bring down transmission to a level where it will no longer pose public health threat, malaria eradication is usually planned at a provincial and national level and this aims for total absence of an autochthonous[5] case in the

[5] Autochthonous malaria refers to non-imported, indigenous case of malaria.

relevant jurisdiction in the next three consecutive years following consolidation phase (World Health Organization, 1999b). Its scope is global and permits curtailing control interventions upon attainment of malaria-free status (Miller et al., 2006). As a result of this global campaign, 37 of the 143 countries that were endemic in 1950 became malaria-free by 1978 (Mendis et al., 2009).

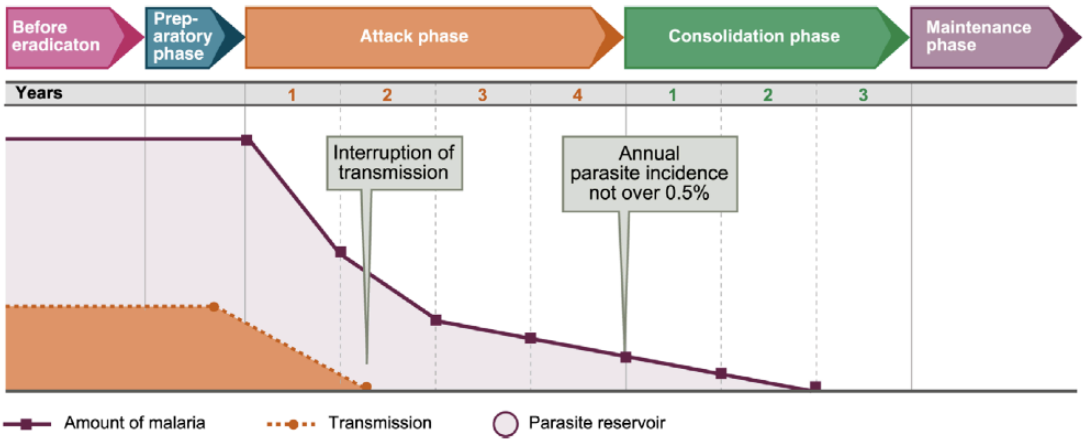


Figure 1.6. The time-limited phases of the GMEP campaign in 1963 (Najera et al., 2011).

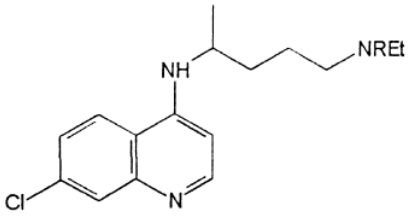
1.5. Chloroquine during the global malaria eradication programme

Rediscovery of chloroquine

Chloroquine was initially synthesised in 1934 at the Elberfeld laboratories of the Bayer I.G. Farbenindustrie A.G. in Germany as the 4-aminoquinoline Resochin (Coatney, 1963). When tested against bird and human malaria in unrecorded trials, Resochin was found to be equally effective to quinacrine (Atabrin)[6] but was mistaken as more toxic so it was shelved in Germany (Coatney, 1963). Resochin was given renewed interest

[6] Quinacrine or mepacrine (brand name Atabrin) was the first synthetic antimalarial developed in Germany in the 1930s. This drug found difficulty in being accepted in the market against the popularity of quinine and quinacrine has side effect of imparting stains on the eyes and skin. COATNEY, G. R. 1963. Pitfalls in a discovery: the chronicle of chloroquine. *Am J Trop Med Hyg,* 12**,** 121-8.

during the WWII United States drug development program when a compound referred to as SN-7618 (SN stands for Survey Number) emerged as the most potent candidate for clinical trial among the 16,000 compounds tested in the span of 4 years and saw that it was actually identical to Resochin (Coatney, 1963, Hahn, 1975, Bruce-Chwatt, 1964). After the clinical trial SN-7618 was found to be safe and tolerable (Loeb et al., 1946). This was named chloroquine and introduced for public use in 1947 (Hahn, 1975).



R = Et, chloroquine
R = H, desethyl chloroquine

Figure 1.7. Structure of chloroquine (Foley and Tilley, 1998).

Mode of Action of Chloroquine

Chloroquine (Figure 1.7) belongs to the type-1 class 4-aminoquinolines of blood schizonticidal drugs targeting intra-erythrocytic stages of *Plasmodium* (Foley and Tilley, 1998). As mentioned in page 11, the asexual stages of *Plasmodium* inside the RBC rely on haemoglobin for survival. *Plasmodium* cannot synthesise amino acids *de novo* and has to use the host RBC's haemoglobin as source of amino acids (Rosenthal et al., 1988, Goldberg et al., 1990). Haemoglobin is taken into the parasite's acidic food vacuole (Elliott et al., 2008) where it undergoes catabolic proteolysis to release amino acids and haem (Goldberg et al., 1990, Goldberg et al., 1991). In the presence of oxygen, haem is released as ferriprotoporphyrin IX, which can inhibit enzymes, destabilise membranes and cause haemolysis (Fitch et al., 1982, Chou and Fitch, 1981). Therefore, it is critical to the survival of *Plasmodium* to dispose haem into non-toxic long insoluble crystalline polymers, the hemozoin (Kapishnikov et al., 2012, Loria et al.,

1999). It was proposed that chloroquine stops this polymerisation process by forming a complex with haem, leading to build-up of toxic haem molecules (Sullivan et al., 1996, Loria et al., 1999). Accumulation of the haem in membranes will alter the permeability of the sodium-potassium transporter on the parasite's membrane(Ginsburg et al., 1998). In addition, the chloroquine-haem complex also prevents haem from detoxifying H_2O_2, which in turn causes peroxidative damage on membranes (Loria et al., 1999).

Chloroquine use during the eradication programme

During the global malaria eradication programme, chloroquine was the most widely used antimalarial to complement indoor residual spraying with DDT (World Health Organization, 1973). Chloroquine distribution and sale was not restricted by governments to health centres and pharmacies so it could be purchased over the counter even in small village shops (Wernsdorfer and Payne, 1991). During the eradication programme, chloroquine was used for individual treatment to achieve radical cure, for presumptive treatment of people showing signs and symptoms of malaria and waiting for confirmation diagnosis, and for mass drug administration, which in some countries was given in medicated salts (World Health Organization, 1973).

1.6. Development and spread chloroquine resistance

The heavy and extensive use of chloroquine in endemic countries put the local sensitive *P. falciparum* population under selection pressure (Wernsdorfer and Kouznetsov, 1980), which led to development of chloroquine resistance. Exposure of the parasite to sub-optimal concentrations of chloroquine caused by non-adherence to treatment duration, improper dosing, and substandard drug properties also contributed to development of resistance (World Health Organization, 1999a, Basco, 2004, Lon et al., 2006, Ursing et al., 2014). Drug resistance is the ability of the parasite to survive and multiply when exposed to a particular drug at a dose equivalent to or

higher than that recommended for parasite clearance in symptomatic malaria, but within the limits of tolerance of the person (World Health Organization, 1973). The spectrum of *P. falciparum* asexual stages responses to chloroquine treatment shown in Table 1 was devised and released by the WHO to assist countries in describing sensitivity and levels of resistance (World Health Organization, 1967).

Table 1.1. *P. falciparum* asexual stages spectrum of responses to schizonticidal drugs (World Health Organization, 1967).

Response	Recommended Symbol	Evidence
Sensitivity	S	Clearance of asexual parasitaemia in 7 days from start of treatment, without recrudescence[7]
Resistance	RI	Clearance of asexual parasitaemia in 7 days from start of treatment but with recrudescence
	RII	Reduction without clearance of asexual parasitaemia
	RIII	No marked reduction of asexual parasitaemia

Chloroquine resistance (CQR) first emerged in 1957 at the Thailand-Cambodia border (Payne, 1987), a focus recognised as a very significant source of CQR spread to Africa (Wootton et al., 2002). In 1960 separate CQR founder events were observed in Colombia and Venezuela in South America (Moore and Lanier, 1961). Meanwhile in the Pacific, CQR was first reported in Papua New Guinea from 1959 to 1961 (Moore and Lanier, 1961) followed by an independent CQR founder event in the Philippines probably in the early 1970s (Chen et al., 2003, Sakihama et al., 2007, Wootton et al., 2002). From the 1960s to the 1980s, CQR rapidly spread throughout South East Asia, America, Pacific and Africa (World Health Organization, 1973, Olatunde, 1977, Hess et al., 1983, Payne, 1987) (Figure 1.8); thus, posing a serious threat to the eradication agenda.

[7] Recrudescence is the appearance of asexual stages of malaria in the blood within a month from the start of treatment.

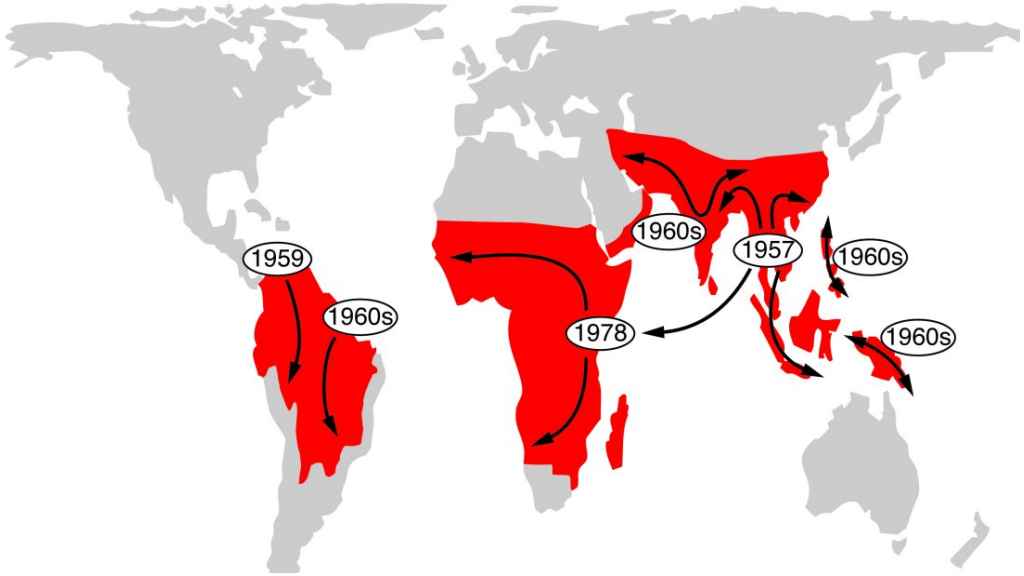


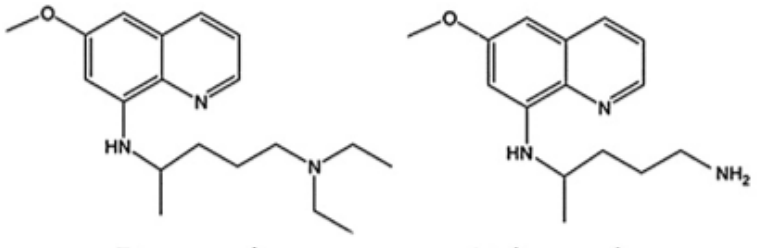
Figure 1.8. Emergence CQR from independent foci in the Thai-Cambodian border, South America, Papua New Guinea, and the Philippines, and its global spread (Payne, 1987, Wootton et al., 2002, Chen et al., 2003, Wellems et al., 2009).

1.7. Use of sulfadoxine-pyrimethamine and development of resistance

Sulfadoxine-pyrimethamine (SP) combination was the replacement drug used to treat chloroquine-resistant *P. falciparum* (Basco et al., 2002). SP acts synergistically (Chulay et al., 1984) by inhibiting two enzymes involved in the *Plasmodium*'s *de novo* folate synthesis pathway necessary for DNA replication. Sulfadoxine inhibits the dihydropteroate synthase (DHPS) (Hyde, 2005) and pyrimethamine inhibits the dihydrofolate reductase (DHFR) (Sirawaraporn, 1998). Resistance to SP was conferred by key mutations in the genes encoding DHPS and DHFR (Plowe et al., 1997). Independent lineages of sulfadoxine resistance evolved in Southeast Asia and South America, with ensuing migration from Asia to East Africa (Mita et al., 2011, Vinayak et al., 2010). Pyrimethamine resistance preceded sulfadoxine resistance (Roper et al., 2003) and this developed from a single origin in Southeast Asia (Nair et al., 2003, Maiga et al., 2007), which eventually spread to Africa. Pyrimethamine resistance also evolved independently in the Pacific (Mita et al., 2007) and in South America (Cortese et al., 2002).

1.8. Primaquine for relapsing malaria and risk of haemolytic anaemia in G6PD-deficienct individuals

Primaquine is an 8-aminoquinoline drug (Figure 1.9) used since the 1950s for prophylactic and radical cure of relapsing *P. vivax* and *P. ovale* malaria (World Health Organization, 2010b, Alving, 1952). It also exhibits gametocytocidal properties against *P. falciparum* (World Health Organization, 2012c, Okebe et al., 2015). The exact mode of action of primaquine is not clear. It was thought to (1) accumulate in the mitochondria of *Plasmodium* where the drug caused changes in inner mitochondrial membrane and thus, disrupting the electron transport chain; (2) produce reactive intermediates that damage erythrocytes (Hill et al., 2006, Waters and Edstein, 2012).



Pamaquine Primaquine

Figure 1.9. Chemical structure of 8-aminoquinolines pamaquine and primaquine (Howes et al., 2013).

Prior to the 1952 approval of primaquine as antimalarial drug by the United States Food and Drug Administration, it was the 8-aminoquinoline pamaquine (plasmoquine) (Figure 1.9), a synthetic derivative of methylene blue that was first used to treat relapsing malaria in 1926 (Sweeney et al., 2004). Pamaquine caused severe acute haemolytic anaemia (AHA), which resulted to death in some 'sensitive' individuals that it was discontinued in 1938 (Howes et al., 2013). During the World War II (1941-1945)

about 95% of the world's quinine supply was curtailed when the Japanese Imperial Army occupied Java in 1942 such that inferior drugs atabrine and pamaquine were used (Joy, 1999). However, this combination triggered 10-fold increase in plasma levels of pamaquine and caused severe AHA such that the United States Surgeon General ordered its withdrawal in 1943 (Baird, 2011). While deployed in the Pacific the Allied Forces mostly suffered from relapsing malaria. The United States Board of Coordination of Malaria Studies acted and screened 14,000 compounds to combat malaria (Condon-Rall, 1994). The search for treatment against relapsing malaria was narrowed to 24 8-aminoquinoline compounds where primaquine was identified as the most likely candidate in 1950 (Howes et al., 2013).

The use of primaquine as antimalarial is limited by its ability to cause haemolytic anemia in some individuals. This AHA was only elucidated when Alving et al. (1956) described the glucose-6-phosphate dehydrogenase (G6PD) deficiency, an X-linked genetic trait that predisposes G6PD-deficient erythrocytes to haemolysis following treatment with primaquine (Marks and Gross, 1959). In the pentose phosphate pathway G6PD catalyses formation of NADH, which reduces oxidised glutathione. In its reduced form glutathione protects the cell from oxidative stress (Berg et al., 2002). In G6PD-deficient erythrocytes, oxidised glutathione is lost and erythrocytes become assaulted by free radicals produced from primaquine metabolites. This process is still unclear. Haemoglobin becomes denatured into insoluble Heinz bodies that stick at the inner membrane of the erythrocyte. The damaged erythrocytes are cleared faster in the spleen; hence, the acute haemolytic anaemia (Beutler, 1994, Bolchoz et al., 2001, Bolchoz et al., 2002, Bowman et al., 2005). To date, the WHO recommends that G6PD deficiency be tested before primaquine is administered to avoid risks of AHA (World Health Organization, 2006, Recht et al., 2014).

1.9. Post-eradication malaria control

Terminating the global malaria eradication programme

The initial optimism at the start of the global malaria eradication waned towards the end of the 1960s. Many developing countries could not sustain the program when international funding was scaled back (World Health Organization, 1999b). Lack of community participation, declining health systems, war and massive population movement further rendered the eradication futile in these countries (Najera et al., 2011). The classic example was Sri Lanka, which was very close to eradicating malaria in 1963 but problems in sustaining eradication led to massive malaria epidemics (Wijesundera Mde, 1988, Najera et al., 1998, Abeyasinghe et al., 2012). In 1969 the global campaign was terminated in favour of long-term malaria control (Orlov and Semashko, 1986). During the 1970s malaria resurgence became a problem in countries where malaria has been substantially reduced, with post-eradication epidemics occurring in India (1973 and 1976) and Turkey (1977) (Ramsdale and Haas, 1978, Sharma, 1996, Najera et al., 1998, Najera, 1999d). In Senegal the emergence of chloroquine resistance increased mortality rate in children less than five years (Trape et al., 1998).

Strengthening global malaria control

Global concern was raised over the worsening malaria situation after the eradication programme was terminated. To address this the 31st World Health Assembly in 1978 adopted a malaria control strategy that encouraged strengthening malaria programmes in endemic countries, provided high priority for control, and where possible in the long-run aimed for an end-goal of malaria eradication (World Health Organization, 1978). Its implementation was hindered by similar problems encountered during the transition of programmes from eradication to control (Najera, 1999a) and remained stagnant. Orlov and Semashko (1986) pointed out that this malaria programme lacked precise strategies such as control strategy based on

stratification and countries confused it with eradication albeit in a non-time limited version. This programme was modified in 1993 under the "Global Malaria Control Strategy", which brought renewed interest in malaria control (World Health Organization, 1993a). It recognised that endemic countries faced different problems (Figure 1.10), and as such control should be based on a primary health care approach (World Health Organization, 1993b, Mendis et al., 2009). The revised strategy aimed to prevent deaths, to reduce sicknesses and socio-economic impact of malaria by using four fundamental elements to strengthen local and national capacities: (1) providing timely diagnosis and treatment; (2) planning and implementing sustainable preventive measures including vector control; (3) detecting, containing and preventing epidemics; and (4) promoting local capacities for basic and applied researches to monitor malaria situation (Najera, 1999a, World Health Organization, 1993b). However, control measures could not cope with increased malaria burden in the wake of rising resistance to low-cost antimalarials (Mbogo et al., 1995, Snow and Marsh, 1995, Trape et al., 1998) that led to a "malaria disaster" in 1999 (White et al., 1999)

The Roll Back Malaria Initiative

The Roll Back Malaria (RBM) Initiative was launched in 1998 by the World Health Organization (WHO), the World Bank, and the United Nations Children's Fund (UNICEF) as a renewed pledge to combat malaria affecting 3.2 billion people in 107 countries and territories (Roll Back Malaria et al., 2005). This initiative identified core strategies for malaria control to reduce malaria burden by 50% in 2010. This included improved and timely access to efficient treatment, augmented use of insecticide-treated nets (ITN) and other means of mosquito control, early recognition and swift response to epidemics, and better preventive and curative measures among pregnant women in endemic regions (Roll Back Malaria et al., 2005).

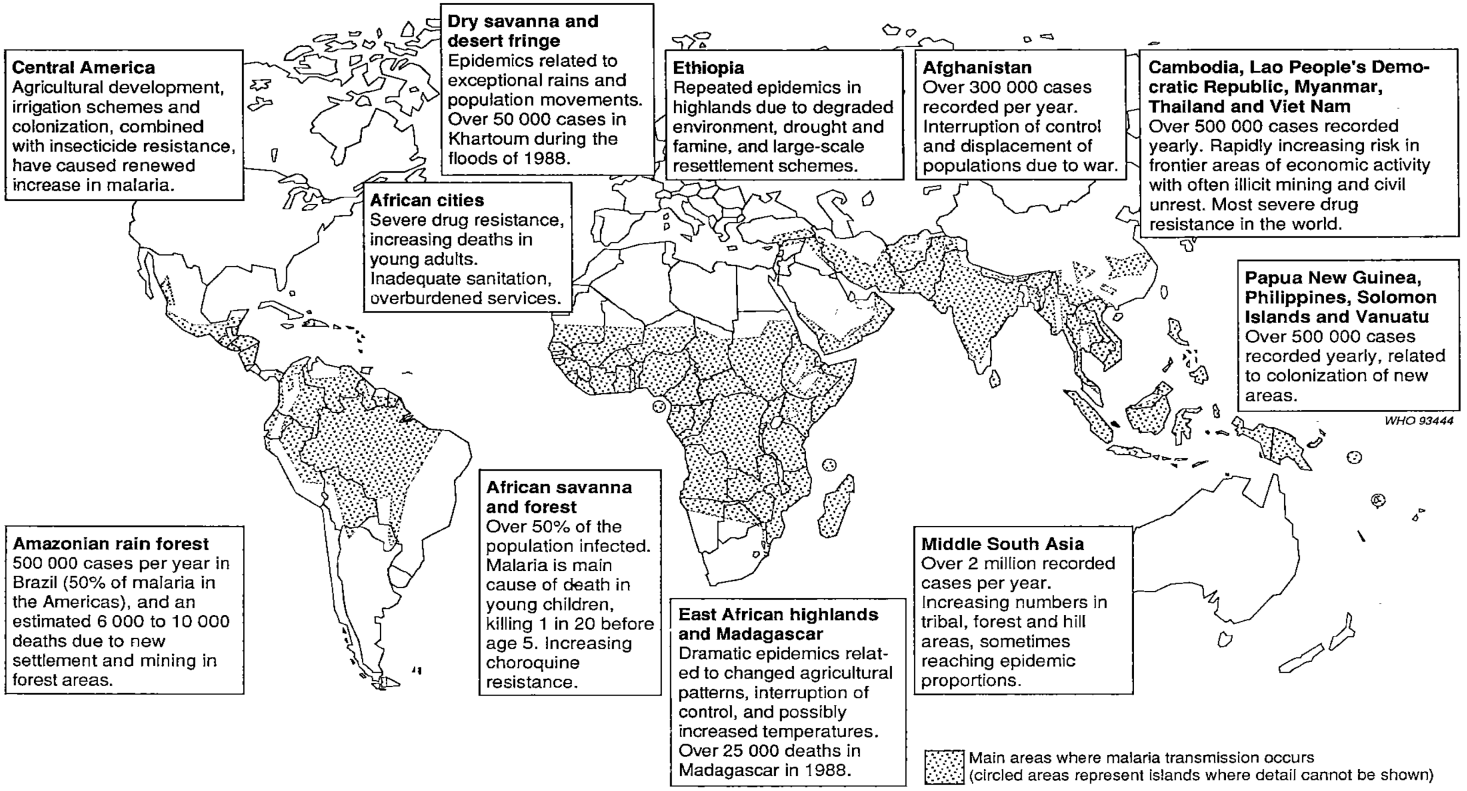


Figure 1.10. Global malaria distribution and problem areas in the 1990s (World Health Organization, 1993b).

1.10. Artemisinin-based combination therapy, the frontline antimalarial of the RBM

In 2001 the WHO recommended that endemic countries change the treatment guidelines to artemisinin-based combination therapy (ACT) when the cure rate of the currently used recommended drug falls below ninety percent (World Health Organization, 2006). The rationale in using artemisinin in combination with a partner drug is two-fold: the combination is more effective and in the rare chance that the parasite developed resistance to either of the two drugs, the partner drug will kill it (White, 1999a, White, 1999b).

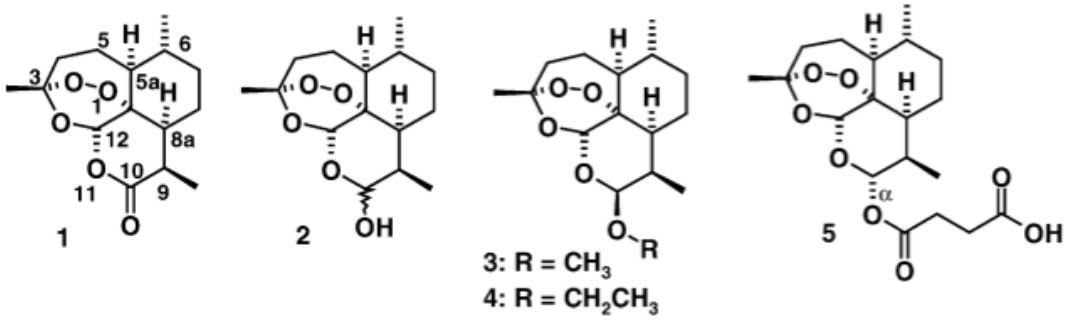


Figure 1.11. Artemisinin (1) and its derivatives: dihydroartemisinin (2), oil-soluble artemether (3) and arteether (4), and water-soluble artesunate (5) (Krishna et al., 2004).

Qinghaosu or artemisinin, which is extracted from the medicinal herb *Artemisia annua* commonly known as sweet wormwood or *qinghao,* has been used to treat fever in China for 2000 years (Tu, 2011, Qinghaosu Antimalaria Coordinating Research Group, 1979). Artemisinin is a sesquiterpene lactone with an endoperoxide group inside a trioxane ring (Figure 1.11, [1]) that is responsible for its antimalarial activity (Pandey et al., 1999). It has very poor aqueous solubility, which is a problem for absorption after being taken orally (Kakran et al., 2011). Artemisinin derivatives artesunate, artemether and arteether are metabolised by the liver cytochrome P450 enzymes to the active compound dihydroartemisinin (DHA) (Figure 1.11, [2-5]) (Svensson and Ashton, 1999). The plasma half-life of the DHA metabolite is only 45 minutes (Ilett et al., 2002), which

prevent resistant parasites from being selected by the drug. Since artemisinins have high recrudescent rates when used as monotherapy, it should be partnered with a longer acting drug to prevent emergence of resistance (Menard et al., 2005). The WHO recommends any of the five ACT combinations for treatment of uncomplicated falciparum malaria: artemether-lumefantrine (AL), artesunate-amodiaquine (AS-AQ), artesunate-mefloquine (AS-MQ), artesunate + sulfadoxine-pyrimethamine (AS+SP), and dihydroartemisinin-piperaquine (DHA-PPQ) (World Health Organization, 2010b).

Mode of action of artemisinins

Artemisinin and its derivatives kill erythrocytic asexual stages of *Plasmodium* (Dondorp et al., 2010, Delves et al., 2012) and reduce gametocyte carriage (Price et al., 1996, Targett et al., 2001). Its peroxide moiety within the 1,2,4 -trioxane system is said to be crucial in its antimalarial activity (Avery et al., 1993, Krishna et al., 2004). Up to now there is no defined consensus on the exact molecular mechanism of action of artemisinin (Olliaro et al., 2001, Meshnick, 2002). It has been suggested that artemisinins exert antimalarial activity by cleaving its endoperoxide bridge to produce highly reactive oxygen species (ROS) (Krungkrai and Yuthavong, 1987, Meshnick et al., 1991). There are two proposed models for opening the peroxide bridge. According to the reductive scission model, low valent ferrous haem or exogenous non-haem iron (Fe^{2+}) binds to artemisinin, cleaves the peroxide bridge and produces oxygen-centered free radicals. The latter generate carbon-centered free radicals by abstracting protons (Paitayatat et al., 1997). In the open peroxide model, addition of a proton (H^{+}) or binding with metal iron results to cleavage of C-O covalent bond and thus, generating an open hydroperoxide that eventually forms peroxy radicals (Haynes et al., 1999). The target of the endoperoxides remains to be debated and the accumulation of artemisinin in the parasite's food vacuole is not clearly understood (O'Neill et al., 2010).

Several studies suggested that artemisinin-derived reactive species alkylate haem (Meshnick et al., 1991, Meshnick, 1994, Hong et al., 1994) and parasite proteins (Asawamahasakda et al., 1994), and that artemisinins inhibit haemoglobin breakdown

and haem polymerisation (Berman and Adams, 1997, Pandey et al., 1999). In addition to these, Li et al. (2005) have demonstrated in yeast models that artemisinins can disrupt the mitochondrial membrane of the parasite by endogenously producing ROS and later on provided direct evidence of this action (Wang et al., 2010). Contrary to these aforementioned models, Eckstein-Ludwig et al. (2003) proposed a different mechanism wherein artemisinins activated by Fe^{2+} to produce carbon-centered free radicals specifically inhibit sarcoplasmic reticulum calcium-dependent ATPase (SERCA) orthologue of *P. falciparum* (PfATP6ase), which is expressed in *Xenopus laevis* oocytes. This claim is further strengthened by demonstrating the effect of artemisinin on calcium homeostasis involving a SERCA analogue in *Toxoplasma gondii* (Nagamune et al., 2007). Thereafter focus is given in elucidating the molecular interaction of artemisinin with PfATP6ase (Jung et al., 2005, Naik et al., 2011), genetic polymorphisms in PfATP6ase that may be selected and reduce the susceptibility of the malaria parasite to artemisinins (Jambou et al., 2005, Jambou et al., 2010, Cui et al., 2012, Uhlemann et al., 2005).

1.11. Global impact of ACT policy and the threat of artemisinin resistance

Since 2001 Asia Pacific countries with levels of resistance to chloroquine and antifolates have adopted ACT into their malaria treatment policy in addition to existing vector control activities such as use of ITN and implementation of IRS (Roll Back Malaria et al., 2005, World Health Organization, 2010a). Malaria morbidity was reduced by 26% from 2000 to 2010, with 1.1 million malaria deaths avoided during this period (World Health Organization, 2012f). This could be attributed to the success of preventive and curative interventions with ACT as the cornerstone of malaria case management (Grueninger and Hamed, 2013). Since artemisinin use is already widespread, regularly monitoring its therapeutic efficacy on the basis of parasitological cure rate is very important to global success against malaria (World Health Organization, 2010b). As things stand at present, artemisinin and its derivatives are the last line of defense against multidrug resistant malaria (Cui and Su, 2009, Enserink, 2010).

Reports of reduced susceptibility of *P. falciparum* to artesunate in the Yunnan Province, China (Yang et al., 2003) and in Western Cambodia (Noedl et al., 2008) and reduced *in vitro* susceptibility to artemether of *P. falciparum* isolates from Cambodia, French Guiana and Senegal (Jambou et al., 2005) warned against the peril of artemisinin resistance. In 2006 the WHO recommended replacing artemisinin monotherapy with ACT (World Health Organization, 2006). In 2009 Dondorp and colleagues (2009) reported reduced *in vivo* susceptibility of *P. falciparum* for artesunate-mefloquine along the Thailand-Cambodian border, which was coincidentally the historical epicentre of chloroquine and pyrimethamine resistance. However, there was no precise definition of artemisinin resistance in 2010 such that reduced parasite susceptibility was treated with caution (Fairhurst et al., 2012). Still, experts concluded these findings portentous that the current ACT might begin to fail (Fairhurst et al., 2012). The World Health Organization (2011d) released the Global Plan for Artemisinin Resistance Containment (GPARC) as a call to action for members of the Roll Back Malaria (RBM) Partnership to act on emergence (Noedl et al., 2008, Dondorp et al., 2009) and potential spread of artemisinin resistance (Maude et al., 2012, Dondorp et al., 2010, Beshir et al., 2013).

In lieu of this threat to malaria control and elimination, there was urgency to identify a molecular marker for global monitoring of artemisinin resistance (Alker et al., 2007, Lim et al., 2009, Witkowski et al., 2010). Ariey et al. (2014) identified mutations in *P. falciparum* kelch protein gene *in* Western Cambodia to be strongly associated with longer parasite survival rate *in vitro* and delayed parasite clearance rate *in vivo* suggesting artemisinin resistance. In a separate study this candidate molecular marker for artemisinin resistance was found among slow clearing *P. falciparum* isolates from mainland Southeast Asia (Ashley et al., 2014).

1.12. Era of global malaria elimination (2007 to present)

Despite the threat of artemisinin resistance along the Thailand-Cambodia border (Dondorp et al., 2011) many countries managed to reduce malaria burden during the latter part of the RBM Partnership (2000-2010) (World Health Organization, 2011e). The WHO backed the call issued by the Bill and Melinda Gates Foundation in 2007 to globally eradicate malaria (The Malaria Elimination Group, 2009). Malaria eradication demands eliminating malaria at a global scale (Chiyaka et al., 2013). In order to achieve malaria elimination there should be complete interruption of mosquito-borne transmission that would reduce local malaria cases to zero, with concerted efforts to prevent its re-establishment from imported cases (World Health Organization, 2011a). This ambitious programme entails finding and treating every malaria infection, removing local transmission through vector control, improving surveillance to prevent re-establishment of malaria, and organising cross-border collaborations (World Health Organization, 2007). In the context of elimination, a malaria case is a person who is diagnosed harbouring *Plasmodium* regardless of the presence or absence of clinical symptoms (World Health Organization, 2012a).

The standard theory of malaria elimination

The feasibility of eliminating malaria in an area depends upon the basic reproduction ratio R_o, which is the expected number of secondary infections caused by one infective host in a naive population (Diekmann et al., 1990). The infection may come from a mosquito vector or human (Roberts and Heesterbeek, 2003). When the source of infection is the mosquito, R_o is described by its vectorial capacity and this number represents the mosquito's human-biting rate and infective life (Garrett-Jones and Shidrawi, 1969). When the source of infection is human, R_o is influenced by sustained asymptomatic infections in the community (Chiyaka et al., 2013). Following the threshold criterion, it is expected that malaria transmission ensues in the population if R_o is greater than one and it stops if R_o is less than one (Diekmann et al., 1990). Implementation of vector control measures such as IRS and ITNs and use of effective

antimalarials leads to reduction in malaria cases represented by the controlled reproduction number R_c, which describes transmission under control in the absence of immunity (Chiyaka et al., 2013, Smith et al., 2013). In order to eliminate malaria in a defined geographic area, R_c must be below one (Smith et al., 2013). There are several challenges to achieving malaria elimination and these are discussed in Section 1.15 in the context of malaria in the Philippines.

Programme advancements towards achieving malaria elimination

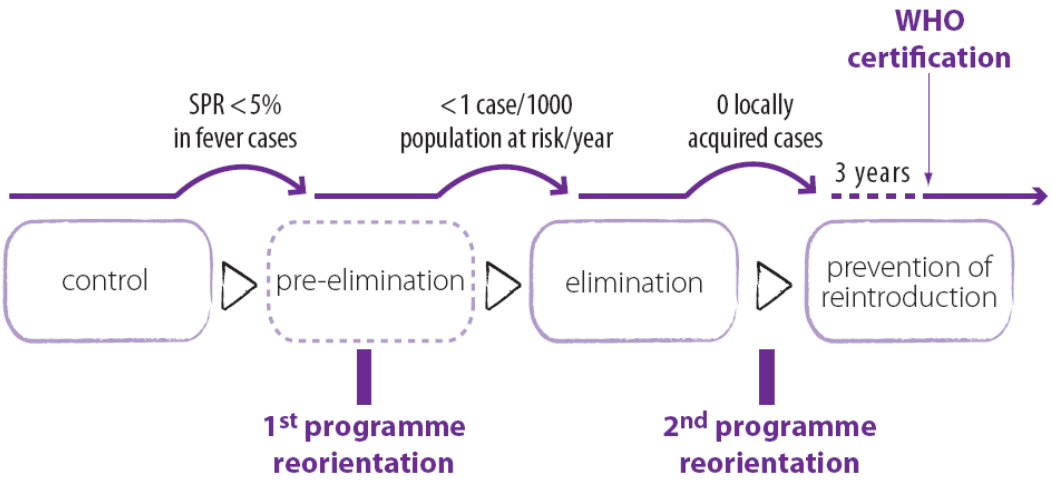


Figure 1.12. Epidemiological milestones towards achieving malaria elimination (World Health Organization, 2007).

Countries with low to medium malaria transmission can transition to pre-elimination when the rate of slide positivity rate (SPR) of febrile cases is less than five percent (World Health Organization, 2007) (Figure 1.12). Pre-elimination is the re-orientation period of a malaria programme between sustained control and elimination, with bolstering of good laboratory and clinical diagnosis coverage, efficient surveillance, reporting and information systems (World Health Organization, 2008). Once a country transitioned to the elimination phase, it has to continue its efforts during sustained control by implementing robust surveillance systems for malaria foci and cases (World Health Organization, 2007, World Health Organization, 2008)

1.13. The Philippines, a malaria-eliminating country

Geography, climate and political subdivisions

The Republic of the Philippines is an archipelagic country of 7,107 islands in the western Pacific Ocean. It has three main island groups namely Luzon, Visayas and Mindanao encompassing a total land area of 115,831 square miles (Figure 1.13) (Bankoff, 2007). The Philippines has a tropical maritime climate with high humidity and abundant rainfall (Balbarino et al., 2003). Based on temperature and rainfall, its climate is divided into dry and wet seasons and the duration of each season depends upon the location in the country. There are provinces in Mindanao where rainfall is evenly distributed throughout the year and where there are no pronounced dry and wet seasons unlike the rest of the Philippines (Figure 1.14) (PAGASA, 2004). Each year an average of 19 to 20 tropical cyclones enter the Philippine area of responsibility (Catane et al., 2012). The Philippines is along the Pacific Ring of Fire, a subduction zone with active volcanic activity that makes the country vulnerable to earthquakes, tsunamis, landslides and volcanic eruptions (The Manila Observatory, 2005). In terms of territorial and political subdivisions, the province or *lalawigan* is the primary local government unit (LGU). The country has 80 provinces grouped into 17 regions. Each province consists of cities or municipalities wherein the village or *barangay* is the smallest political unit (Department of Interior and Local Government Philippines, 1991). Provinces are grouped into Regions I to XII and the National Capital Region is the seat of national government (Figure 1.13).

Figure 1.13. Map of the Republic of the Philippines showing the country's administrative boundaries (inset is the National Capital Region) (Department of Health Philippines et al., 2014).

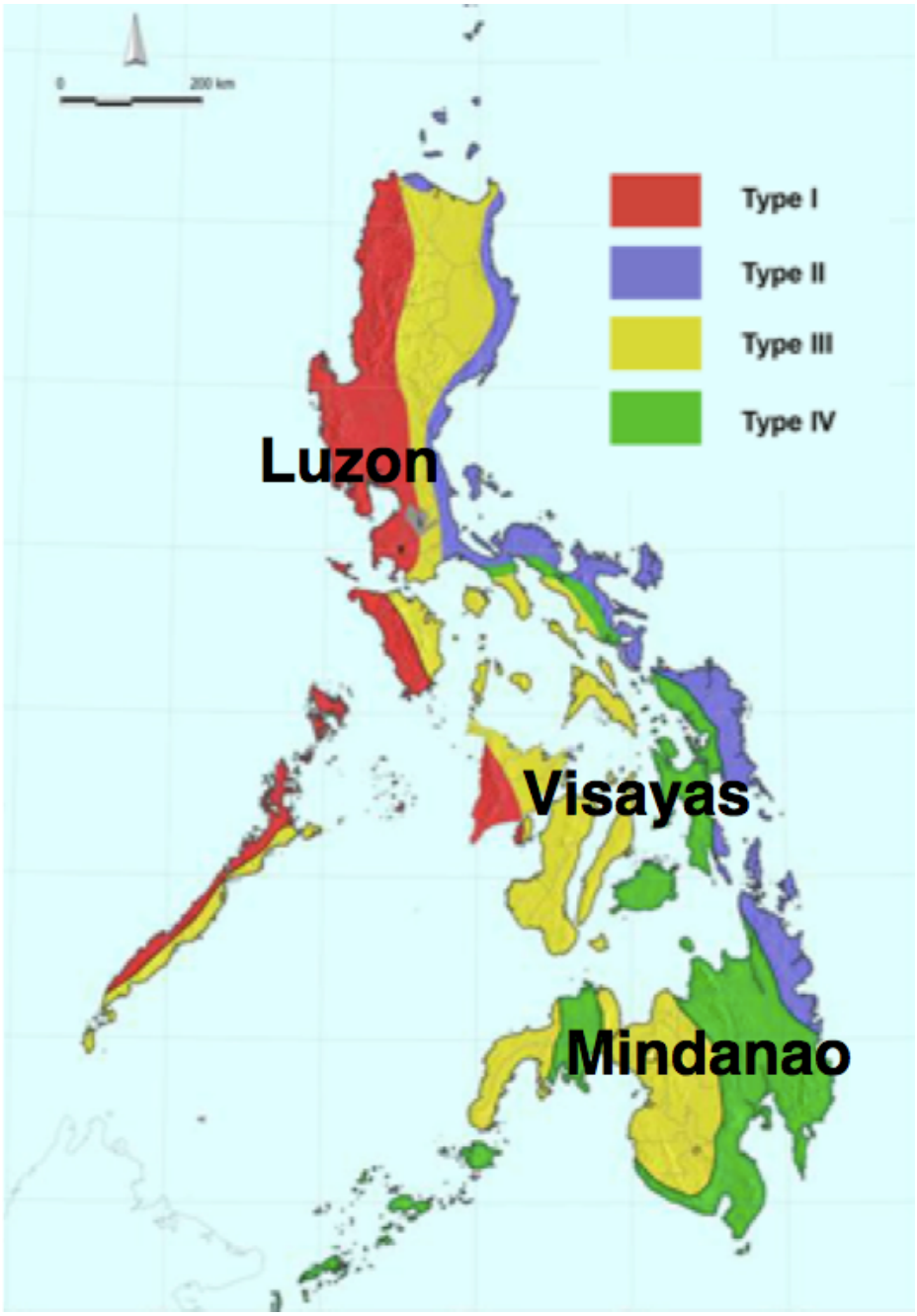


Figure 1.14. Philippine climate map based on modified Coronas classification: Type I areas in red have dry season from November to April and abundant rain for the rest of the year; type II areas in violet has wet season all year round and maximum rainfall from December to February; type III areas in yellow have very short dry season lasting for one to three months followed by rainfall all year; and type IV areas in green has even rainfall all year (PAGASA, 2004).

Current malaria status

The Philippines is a malaria endemic country in the Western Pacific Region that set a goal to eliminate malaria by 2020 (Department of Health Philippines, 2011). This malaria elimination initiative was launched in 2007 and procedures for declaring malaria-free provinces were formalised in 2011 as part of the National Malaria Medium Term Development Plan 2011-2016 (Department of Health Philippines, 2011, Department of Health Philippines et al., 2014). There was 80% reduction in reported malaria cases by microscopy from year 2000 to 2012 from scaling up vector control through wider coverage of IRS and ITN and adopting the artemether-lumefantrine (AL) for treatment of uncomplicated falciparum malaria in 2009 (Liu et al., 2013, World Health Organization, 2013). In 2013 7.1 million Filipinos lived in high transmission areas, 71.6 million in low transmission areas, and 19.9 million in malaria-free areas (Figure 1.15) (World Health Organization, 2014b). Overall, the country is currently at the control phase while undergoing subnational malaria elimination (Department of Health Philippines et al., 2014).

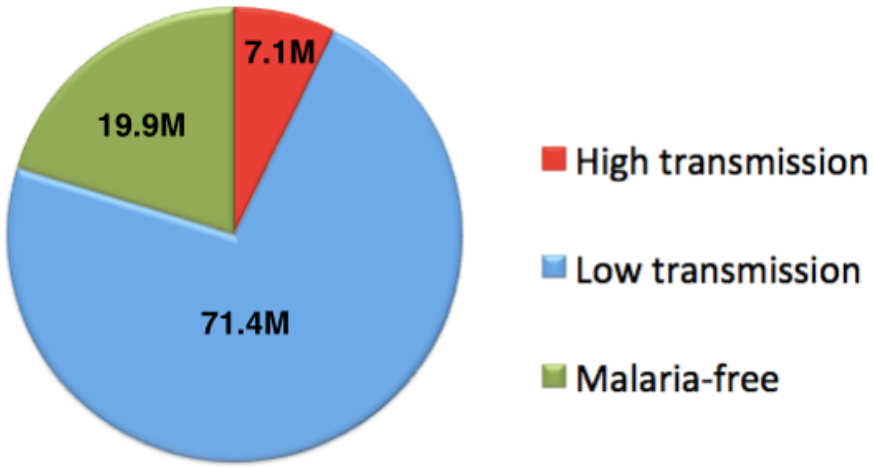


Figure 1.15. Distribution of 98.4 million Filipinos in areas of varying malaria transmission in the Philippines in 2013. Data source (World Health Organization, 2014b)

Philippine malaria vectors

The *Anopheles* species transmitting malaria in the Philippines are *An. minimus flavirostris* Ludlow (1914), *An. mangyanus* Banks (1907), *An. maculatus* Theobald (1907), *An. balabacensis* Baisas (1936) and *An. litoralis* King (1932) (Asinas, 1993, World Health Organization, 2014b, Torres et al., 2000). The distribution of these species and their most likely habitats are presented in Figures 1.16 and 1.17 respectively.

An. minimus flavirostris is the main vector of malaria in the Philippines (Foley et al., 2002) and this species is widely distributed in many endemic provinces (Figure 1.16). *An. minimus flavirostris* is anthropophilic and prefers to stay outdoors (exophilic) but feeds indoor mostly at night (Catangui et al., 1985, Schultz, 1993). This species prefers clear shaded running water along foothills, hillsides and forest edges although it is adaptable to low lands under different climatic conditions. In addition, this species transmits malaria below 2,000 feet (Salazar et al., 1988, Asinas, 1993).

An. mangyanus, a native species to the Philippines, is found in but not limited to Mindoro and Mindanao Islands (Figure 1.16). This also breeds in slow moving streams along foothills, hilly zones and forest edges like *An. flavirostris* but *An. Mangyanus* has limited flight range (Figure 1.16) (Catangui et al., 1985, Salazar et al., 1988). On the other hand *An. maculatus* is said to transmit malaria at higher altitudes with preference for outdoor feeding and cohabits with *An. flavirostris* in well-lit parts of streams (Russell, 1933, Salazar et al., 1988, Torres et al., 1997). Meanwhile, *An. balabacensis* is a sylvatic species confined in deep forests and its three variant species are found each in Mindanao, western islands including Palawan and Balabac Islands, and lastly in Luzon island (Salazar et al., 1988, Torres et al., 1997). *An. balabacensis* is highly anthropophilic but also feeds on monkeys making it a very good vector of simian malaria (Asinas, 1993). *An. litoralis* breeds in brackish water along coastal areas, lagoons and coral reefs (Salazar et al., 1988, Torres et al., 1997). In regions of the Philippines with wet and dry seasons, drying up of streams during the dry season and flushing of streams during the rainy season could interrupt transmission (Barber et al.,

1915, Russell and Santiago, 1934, Asinas, 1993). At the end of the rainy season, it was expected that transmission would ensue from increase of *Anopheline* vectors but in areas where there were no marked dry and wet seasons malaria transmission would be perennial (Ejercito et al., 1954, Nakabayashi et al., 1974, Department of Health Philippines, 2011).

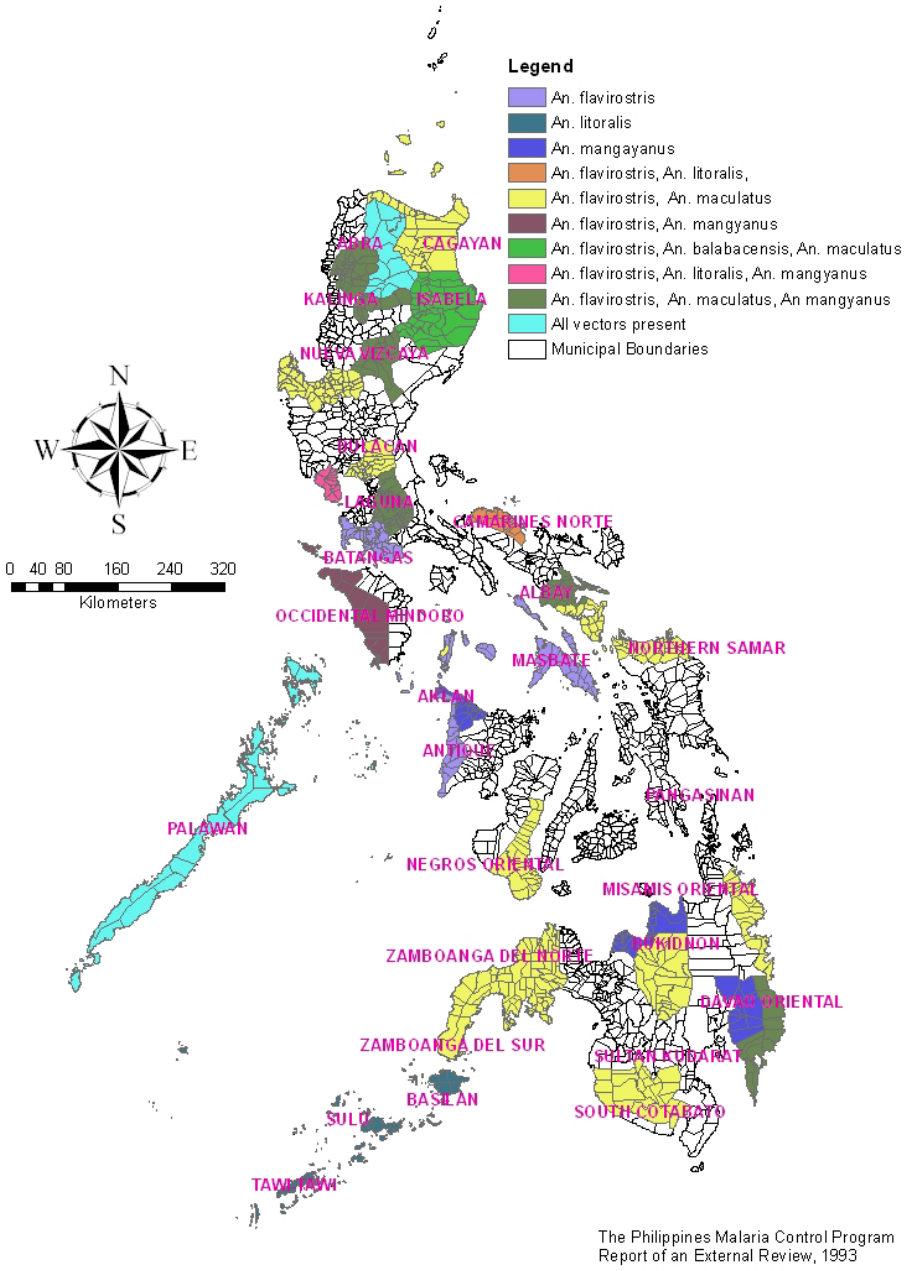


Figure 1.16. Distribution of malaria vectors in the Philippines (Department of Health Philippines et al., 2014).

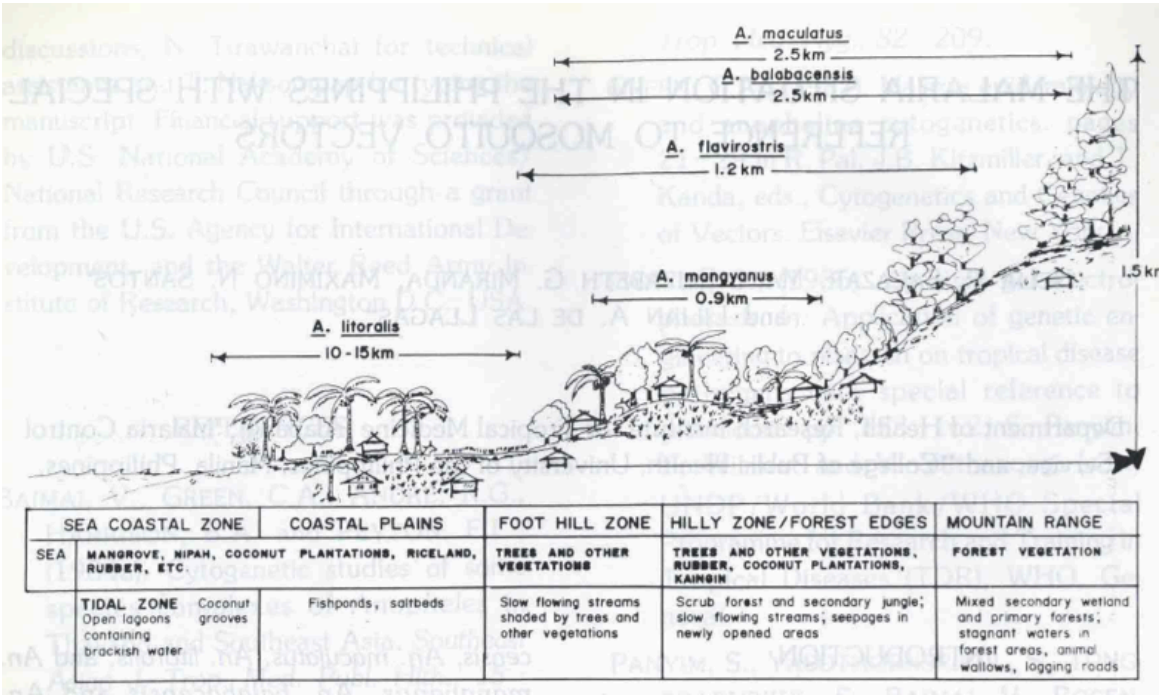


Figure 1.17. Habitat distribution of Philippine malaria vectors (Salazar et al., 1988).

Transmission limits of Philippine malaria

In 2012 approximately 69% of malaria cases in the Philippines was caused by *P. falciparum* while 31% of cases was caused by *P. vivax* (World Health Organization, 2013). These two species overlap in endemic provinces of the Philippines (Figures 1.18 and 1.19). Other *Plasmodium* species constitute less than 1% of cases in 2009 (World Health Organization, 2010g). *P. malariae* has been reported in Sulu, Palawan, Mindanao and Luzon (Cabrera and Arambulo, 1977) while *P. ovale* is uncommon in the Philippines. Two indigenous cases of *P. ovale* have been reported from the Palawan Islands in 1968 (Alves et al., 1968). *P. ovale* may still be present in the Philippines but further studies are needed to confirm this as *P. ovale* it is not routinely screened by microscopy in primary health care units. On the other hand, human infections with the zoonotic *P. knowlesi* have been reported in the Palawan Islands (Luchavez et al., 2008)

but the extent of its burden and public health threat in the Philippines is yet to be determined.

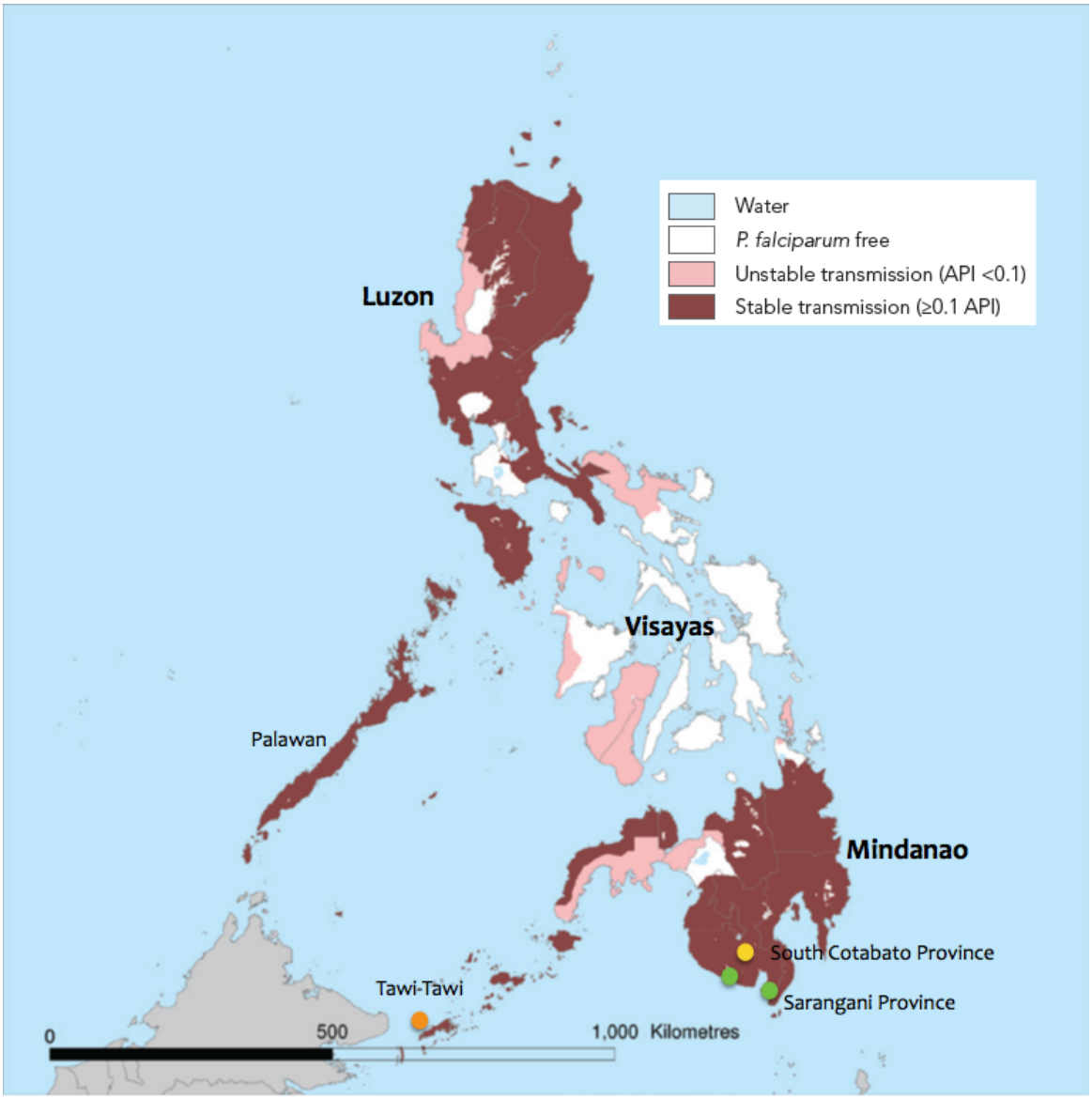


Figure 1.18. Spatial limits of *P. falciparum* transmission in the Philippines in 2010 (Gething et al., 2011, The Global Health Group, 2013). Map was modified to show study sites in Mindanao.

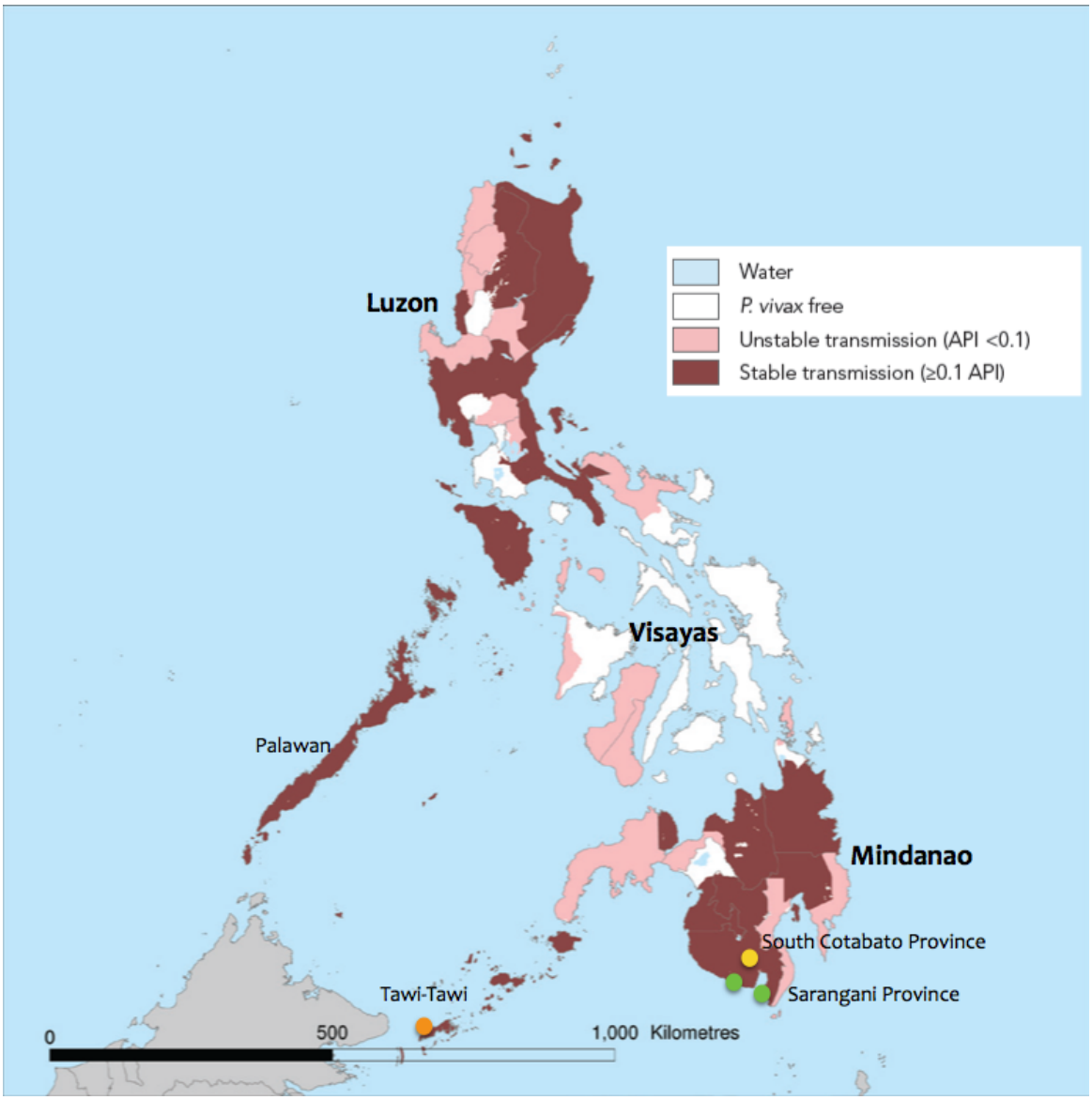


Figure 1.19. Spatial limits of *P. vivax* transmission in the Philippines in 2010 (Gething et al., 2012, The Global Health Group, 2013). Map was modified to show study sites in Mindanao.

Stratification of malaria-endemic provinces in the Philippines

The Philippines provinces are classified based on levels of endemicity (Table 1.2) and the distribution of these are shown in Figure 1.20. In 2013 57 provinces remained endemic for malaria while 23 provinces were declared malaria-free (Philippines National Malaria Program, 2014f).

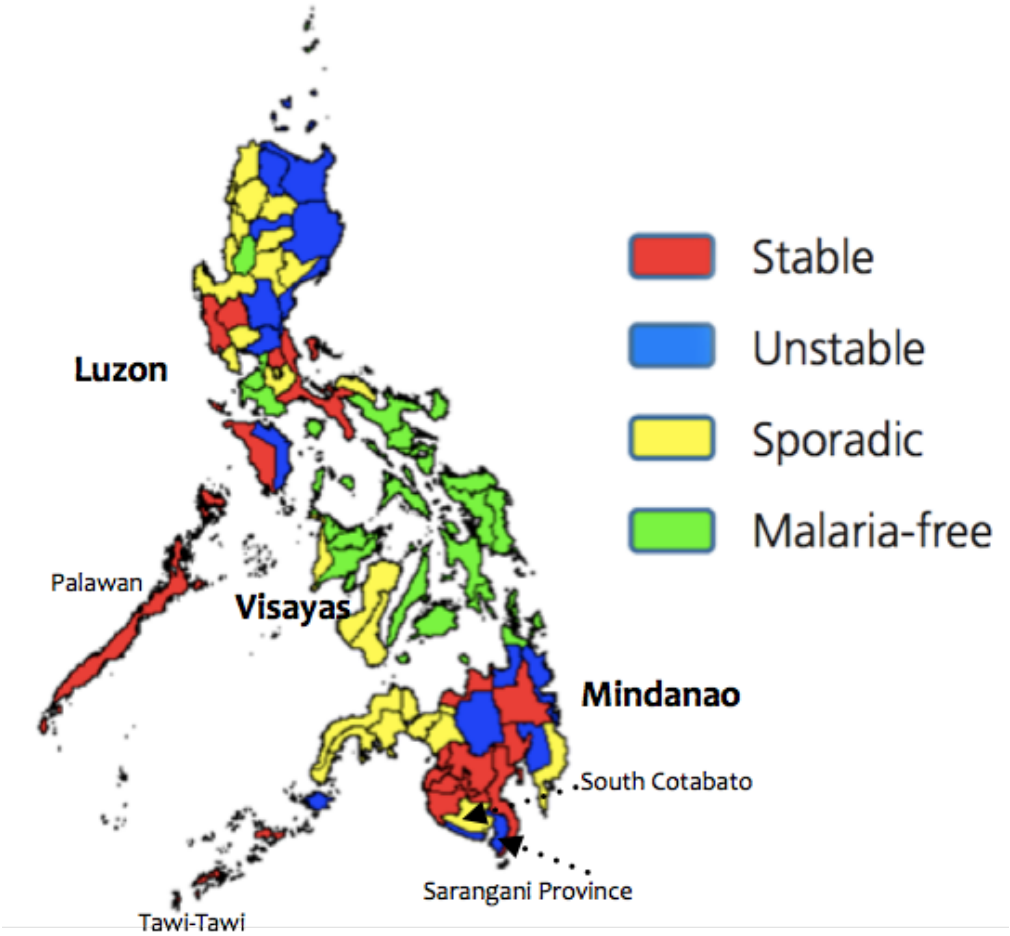


Figure 1.20. Endemicity of malaria in provinces of the Philippines in 2013 (Department of Health Philippines et al., 2014)

Table 1.2. Malaria stratification of endemic provinces in the Philippines in 2013 (Philippines National Malaria Program, 2014f).

Stratum	Description[8]	No. of Provinces
1. Stable Risk	with at least one village or *barangay* having one indigenous case in a month for 6 months or more, at any time in the past three years (2010-2012)	24
1.1. High	with > 1,000 malaria cases	(2)[9]
1.2. Moderate	with 100 to < 1,000 malaria cases	(9)
1.3. Low	with < 100 cases	(13)
2. Unstable Risk	with at least one *barangay* experiencing incessant incidence of at least one indigenous malaria case within a month, for less than 6 months in the past three years (2010-2012)	12
3. Epidemic risk or sporadic risk	with at least 1 *barangay* with at least one indigenous malaria case at any time in the past 5 years	17
4. Malaria-free	without indigenous malaria in the last 5 years even in the presence of mosquito vector	27
Total		**80**

[8] The description of each stratum was based from the DEPARTMENT OF HEALTH PHILIPPINES 2011. Malaria Medium Term Development Plan 2011-2016. Manila: National Malaria Control Program. This 2013 stratification was based on 2010-2012 data to reflect changes in malaria endemicity after 2009.

[9] Figures in parentheses fall under "Stable Risk" stratum.

1.14. Philippines progress from control to elimination

Malaria in pre-Spanish Philippines

When Magellan arrived in 1521, malaria was already in the Philippines (Newson, 1999). The period when malaria became established in the Islands[10] was not known but it could be more recent because of the limited genetic resistance to malaria such as glucose-6-phosphate dehydrogenase (G6PD) deficiency and haemoglobinopathies among Filipinos (Newson, 1999). References to malaria in the Philippines were scarce during the Spanish colonisation (1521-1898) and almost none in Mindanao, which did not succumb to Spanish conquest (Newson, 2011). It was only after 1898 when Spain ceded claim over the Philippines to the Americans that malaria became well documented in the Islands (Russell, 1934).

Philippine malaria control during the American colonisation

When the Americans arrived in 1898 malaria was already serious and widespread in the Islands (Heiser, 1912, Haughwout, 1918). Tertian and "aestivo-autumnal" malarial fevers were observed in the 1900 (Flexner and Barker, 1900). During this time quinine was used for prophylaxis and treatment yet, the death toll for malaria was approximately 212,000 from 1902 to 1903 (Russell, 1934). This prompted a mandate for compulsory use of bed net or *mosquitero* in all malarious areas in 1904 since this was the only available means of control for average households (Russell, 1934). In 1906 the Bureau of Health[11] distributed free quinines to malarious districts, which later on was found to provide palliative relief rather than control malaria (Russell, 1936). Reduction in the mortality rates due to malaria subsequent to 1906 (Figure

[10] The Philippines was referred to as "*Las Islas Filipinas*" during the Spanish colonisation in honour of King Phillip II of Spain, and the "Islands" during American colonisation. When it attained independence in 1946 its official name became the Republic of the Philippines.

[11] In 1901 Philippine Commission created the Bureau of Health, which was known as Philippine Health Service from 1915-1933, and thereafter referred to as the Philippines Department of Health (DOH).

1.21) transpired after improving the quality of *mosquiteros* used in malarious districts (Russell, 1934) . There were efforts to grow *Cinchona* in the Philippines in 1912 to cope with the growing demand for quinine, which was still costly for average Filipinos and has to be imported into the country (Marañon et al., 1935). The alkaloid extract referred to as Philippine "Totaquina[12]", which was found to be equally effective to quinine sulphate was envisaged to eventually dominate Philippine market to replace costly antimalarials quinine, plasmochin, and atabrine (Republic of the Philippines, 1947, Malaria Commission, 1935). In 1913 the larvivorous fish *Gambusia affinis* (Baird and Girard) was brought to the Philippines from Honolulu, Hawaii and introduced in local streams and ponds to feed on mosquito larvae (Malaria Commission, 1935). Unfortunately, this was not successful because *Gambusia* was eaten by the local mudfish or *dalag* (Russell, 1936).

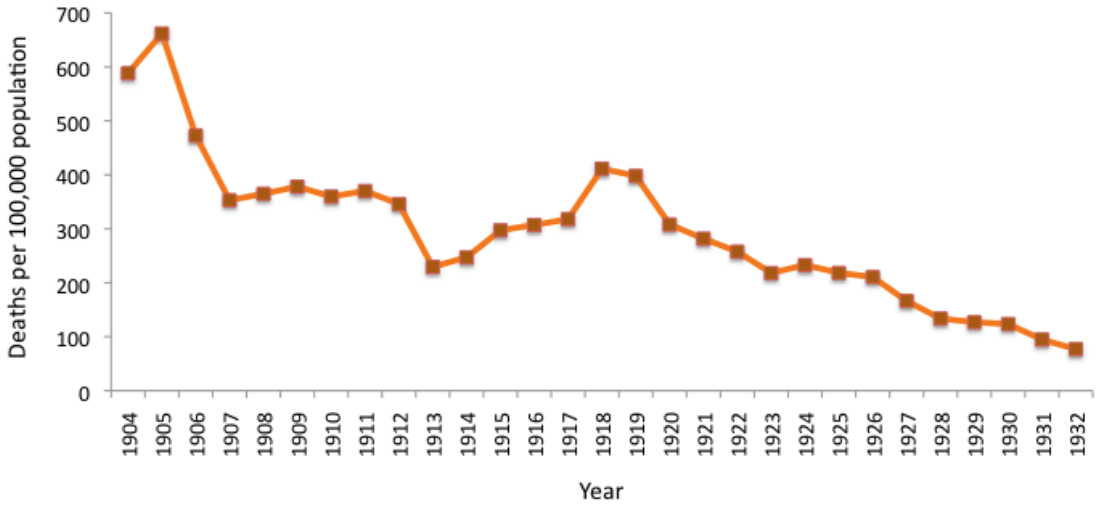


Figure 1.21. Malaria-attributed deaths in the Philippines from 1904 to 1932. Population estimates in 1900 was 7,000,000 and in 1931 about 13,000,000 (World Health Organization, 2012d, Russell, 1934).

[12] The Philippine Commonwealth Act No. 646 approved in July 11, 1941 created the establishment of *Cinchona* plantations in public forestlands for production of Totaquina. However, it was not clear in literature whether Philippine "Totaquina" flourished in the market after the Second World War.

An important milestone to Philippine malaria control occurred when Walker and Barber (1914) found that the chief mosquito vectors of malaria in the Philippines belonging to the *funestus-minimus* subgroup of *Anopheles* preferred the shaded, clear running streams and creeks along foothills. A year after, Barber (1915) suggested that the best way to control Philippine malaria was to destroy the larvae of these mosquitoes in breeding sites. However, the significance of Walker (1914) and Barber's (1915) findings was only realised 10 years after because of the persistent classical idea that mosquitoes transmitting malaria in the Philippines bred in swamps.

In 1921 the International Health Division of the Rockefeller Foundation took interest in Philippine malaria control and promoted the use of Paris green, which proved to be an effective method in destroying mosquito larvae (The Rockefeller Foundation, 1926). The foundation also organised and developed the Malaria Control Service before transferring this to the Philippine Health Service in 1926 to supervise larviciding with Paris green (World Health Organization, 2012d). Despite central government efforts, malaria control was not sustained at the community level (Russell, 1934). The World War II disrupted control activities such that there were estimated 4 million malaria cases in the Philippines by the end of the war (Ejercito et al., 1954)

Rehabilitation of the Philippine Malaria Control Program

Malaria became the leading cause of morbidity in the Philippines after the WWII (Asinas, 1993). This compelled the United States Public Health Service to organise the Philippine Public Health Service Rehabilitation Programme, which became active from 1946 to 1950 (Ejercito et al., 1954, Cabrera and Arambulo, 1977). The programme conducted control activities and pilot tested DDT-treated sawdust, which was found to be effective in reducing malaria burden (Ejercito et al., 1954). In 1947 the Commonwealth President Manuel Roxas signed into law the creation of the Department of Health (DOH) (Republic of the Philippines, 1947). The DOH Malaria Control Division partnered with the United States Operations Mission to the Philippines to form the "Six Year Philippine American Program for Malaria Control in the Philippines" in 1953 (Ejercito et al., 1954). This programme aimed to reduce

malaria to a level where it would no longer be a public health threat and to assist the Philippine government to use its own resources to sustain control activities in the long run (Espino et al., 2004). The new programme employed residual spraying using DDT and chloroquine to reduce malaria burden, but chloroquine was used solely for controlling malaria in areas not reached by DDT spraying (Ejercito et al., 1954). The success of DDT and chloroquine in reducing malaria-attributed deaths and morbidity (Figure 1.22) encouraged national optimism such that in 1956 the Philippines shifted gears towards malaria eradication (Cabrera and Arambulo, 1977, Asinas, 1993).

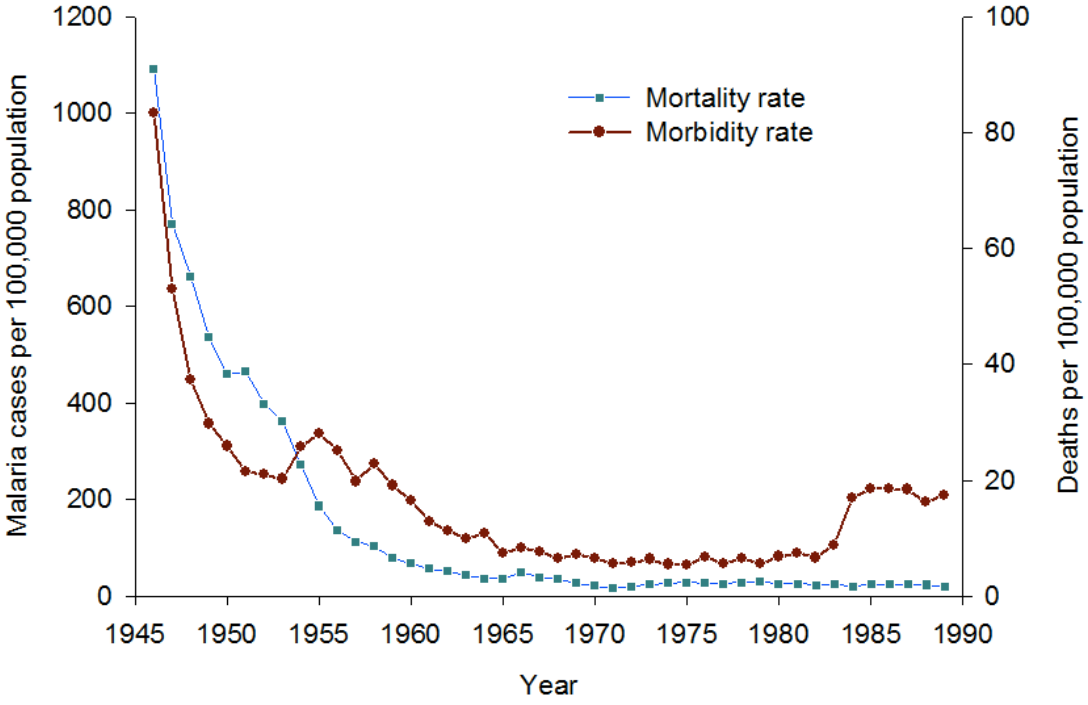


Figure 1.22. Malaria-related cases and deaths from 1946 to 1989. Data source: (Asinas, 1993). Important events in the Philippines malaria program during this period include shifting to eradication programme in 1956, decentralisation of health systems in 1959, its re-centralisation in 1966 and second decentralisation in 1983 (Espino et al., 2004).

Malaria eradication during structural reorganisation of the Philippine health system

Progress towards eradicating malaria in the Philippines was hampered when the Malaria Control Program (MCP) was decentralised and placed under the control of the Regional Health Offices in 1959 (Espino et al., 2004). This resulted to decline of operations in many provinces because of inadequate supervision and lack of funding from local government units (LGU) (Asinas, 1993, Espino et al., 2004). As a result there was resurgence of malaria in provinces where malaria was previously brought down and thus, dimming the prospect of eradication (Asinas, 1993). The worsening malaria conditions prompted the Philippine Congress to enact the Malaria Eradication Act (Republic Act No. 4832) in 1966 to recentralise the malaria program and to provide funding for its activities, with external assistance from the United States Agency for International Development (USAID) and the WHO (Cabrera and Arambulo, 1977, Asinas, 1993). When the Malaria Eradication Act was enforced, the Philippines adopted the WHO list of recommendations for eradication (World Health Organization, 1957). However, the programme faced operational challenges in hard to reach communities and in coping with population mobility, habits of indigenous people and emergence of chloroquine resistance (Cabrera and Arambulo, 1977). The programme further suffered from reduced logistic support from the central government and insufficient funding when external support from the WHO and the USAID was scaled back in 1973 (Asinas, 1993).

Reverting from eradication to malaria control

The Philippines adopted the primary health care (PHC) approach to the delivery of health services in 1981 and this was integrated into the malaria control system (Gomes and Salazar, 1990, Espino et al., 2004). The Philippine malaria service was decentralised for the second time in 1983 and eradication activities were terminated in favour of malaria control (Espino et al., 2004). It was against this background that the devolution of national government services in the Philippines took effect under the Philippines Local Government Code (LGC) of 1991 (Grundy et al., 2003). The principle behind the government decentralisation was to allow LGUs to decide on the needs of

its immediate sectors and to deliver services based on these needs (Lakshminarayanan, 2003). During this period malaria control operated at a semi-vertical programme with the DOH central unit involved in developing policies while each LGU implemented vector control, case finding and treatment at the community level (Asinas, 1993, Espino et al., 2004). Chloroquine and sulfadoxine-pyrimethamine, which could be availed from the free market, were in use in the Philippines at this period (Gomes and Salazar, 1990, World Health Organization, 2005) despite increasing reports of resistance (Buck et al., 1983, Watt et al., 1987c, Baird et al., 1996a). On the other hand, primaquine was controlled by the government and could only be availed from rural health units (RHU) (Gomes and Salazar, 1990). This must have prevented local *P. vivax* from being selected by primaquine pressure. In 2006 the Philippines began distributing ITN and LLITN free of charge as cost-effective means of controlling malaria (World Health Organization, 2013). The nationwide effort reduced the number of malaria cases by 82.7% from 1990 to 2012 (Figure 1.23) (World Health Organization, 2013).

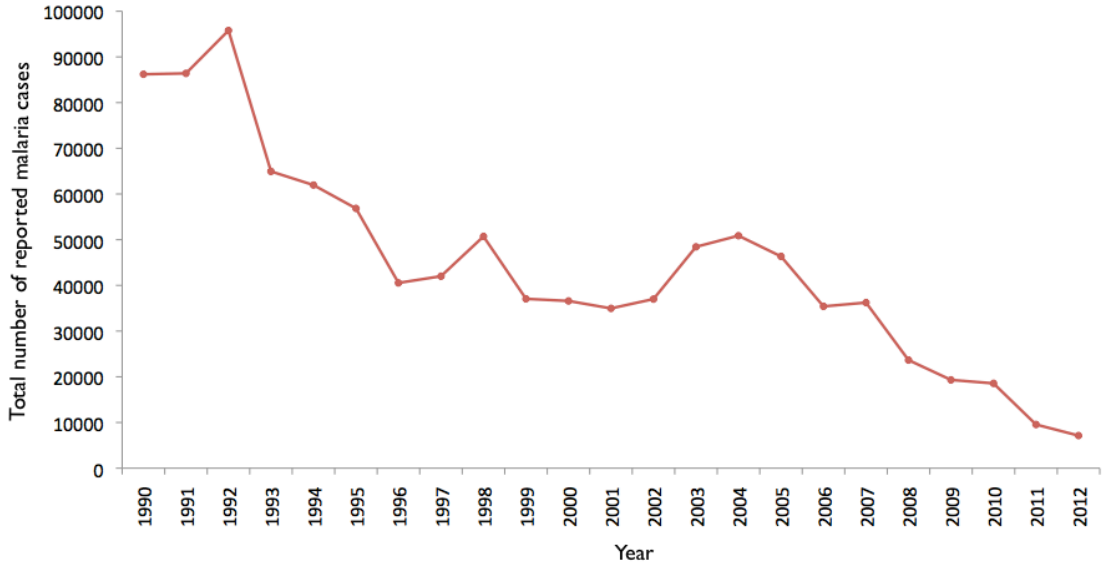


Figure 1.23. Reported malaria cases in the Philippines from 1990 to 2012. Data source: (World Health Organization, 2013).

From malaria control to elimination

In 2001 RBM activities started in the Philippines, initially targeting highly endemic municipalities in southern Mindanao and concentrating on establishing *barangay* diagnostic centres, training health workers and procuring essential malaria supplies (Roll Back Malaria, 2005). The increase in morbidity rates from 2002 to 2005 (Figure 1.23) could be attributed to improved diagnostics deployed in *barangays*. It was also in 2002 that chloroquine and sulfadoxine-pyrimethamine combination (CQ-SP) became the first-line treatment for uncomplicated falciparum malaria in the Philippines, while chloroquine and primaquine remained the treatment of choice for vivax malaria (World Health Organization, 2005). The MCP has received external support from the Global Fund from 2002 to 2010. This funding allowed the programme to provide universal access to diagnostics and malaria treatment in many *barangays*, to scale up vector control largely by ITN and long-lasting ITN in endemic provinces, and to intensify local malaria control and surveillance (Roll Back Malaria, 2005, Tropical Disease Foundation, 2008, Department of Health Philippines, 2011).

The malaria elimination initiative began in the Philippines in 2007 when the DOH launched a phased strategy to eventually eliminate malaria nationally by 2020 (Department of Health Philippines, 2011). The 80 provinces were classified based on level of malaria endemicity as stable risk, unstable risk or malaria-free to determine the extent of interventions required (Department of Health Philippines, 2011). There was a dramatic 78% reduction in malaria cases from 1990 to 2010 (World Health Organization, 2013). There were 23 provinces declared malaria-free in 2010, which added optimism that malaria could be eliminated in the Philippines (Department of Health Philippines, 2011). The goal of the 2011-2016 Malaria-Medium Term Development Program (2011) was to hasten the transition from sustained control to elimination.

1.15. Challenges to Philippine malaria elimination

There are several challenges to overcome in order to achieve malaria elimination in the Philippines. First is the threat of antimalarial drug resistance. The ACT policy in the Philippines has been introduced in 2009 (World Health Organization, 2010g) against a genetic background of *P. falciparum* that has undergone years of selection pressure from chloroquine (Nakabayashi et al., 1974, Smrkovski et al., 1982, Baird et al., 1996a), amodiaquine (Watt et al., 1987a) and sulfadoxine-pyrimethamine (Watt et al., 1987c). Chloroquine and primaquine are the treatment of choice for *P. vivax* in the Philippines (Department of Health Philippines, 2010). Drug efficiency monitoring in the Palawan islands, The Philippines indicated chloroquine-sensitive *P. vivax* (Baird et al., 1996a, World Health Organization, 2005) but there were no data on other endemic provinces to suggest whether or not *P. vivax* has developed resistance after more than 60 years of chloroquine pressure. Second, there is uncertainty in the extent of public health threat of *P. vivax* and other non-falciparum malaria species in the Philippines. In decades of *P. falciparum* dominance in the Philippines, the relative threat of *P. vivax* has been underappreciated. On the other hand, human infections with the zoonotic *P. knowlesi* have been reported in the Philippines (Luchavez et al., 2008) and many countries in Asia (Singh et al., 2004, Jongwutiwes et al., 2004, Ng et al., 2008, Van den Eede et al., 2009) but information regarding *P. knowlesi* or *P. malariae* and *P. ovale* spp. is very inadequate in the Philippines. Third, the available diagnostics in local communities such as microscopy and RDT (Department of Health Philippines, 2010) may lose efficacy with asymptomatic malaria infections (Ouedraogo et al., 2009, Sturrock et al., 2013) and fail to diagnose these people who are capable of perpetuating transmission (Dinko et al., 2013). Fourth, there is limited information on malaria in geographically isolated and depressed areas and in areas of conflict in Mindanao (The Global Health Group, 2013), which can influence the spread of malaria across endemic provinces.

CHAPTER 2: Aims and Plans of the Study

2.1. Justifications of the study

The Philippines has launched an ambitious goal to eliminate malaria by 2020. An important lesson from the failed global malaria eradication attempt from 1954 to 1969 is to base policies and strategies on the local epidemiology of malaria. There is a large gap in the Philippines in this aspect as very little is known about epidemiology of malaria in many endemic provinces. From the 1970s researches about malaria in the Philippines focused on the Palawan Island. This was understandable considering the high mortality and morbidity rates in Palawan compared to other provinces. Still, the malaria burden coming from the 22 (42%) endemic provinces in Mindanao could not be taken lightly if the nation is zealous in its goal of eliminating malaria. One reason for limited data in malaria epidemiology in Mindanao is that information-gathering activities have not been sustained and only two of its 22 provinces have functional Provincial Epidemiology and Surveillance Units as of 2009. The second and probably the foremost reason was the longstanding history of armed conflicts in Mindanao. These armed conflicts between local rebel groups and the Philippine government have resulted in evacuations and population movements across endemic provinces on the Mindanao islands. Official malaria prevalence data from provinces with ongoing insurgencies might not reflect the true burden of disease in the population. The provision of evidence-based epidemiological data for Mindanao is very important in order to ascertain the degree of interventions needed to progress towards malaria elimination.

In addition to this, the epidemiology of malaria may be changing across endemic provinces in Mindanao as the Philippines managed to reduce more than 75% of its national malaria burden in the last decade. In effect, transmission may be restricted to areas that were hardly reached by control measures such as indigenous communities in the uplands. The predicament to this would be the country's limited ability to locate residual foci of malaria transmission and to diagnose people with subpatent malaria infection. The available field diagnostics microscopy and RDT would not be able to find malaria infection if the parasite densities in infected humans were below detection

limit. The indigenous tribes, for instance, could transmit or re-introduce malaria in the lowlands where herd immunity has waned after years of sustained malaria control. Thus, the hidden burden of undiagnosed malaria infections in these residual foci would continually perpetuate transmission. The hidden malaria burden could be caused by any *Plasmodium* species continually circulating in the population. It would be significant to the country's bid for malaria elimination to locate these residual foci and address the hidden *Plasmodium* burden to interrupt transmission.

Since the Philippines officially adopted ACT-based policy in 2009, it would be vital to know the genetic background of *P. falciparum* that might limit ACT's usefulness in the country. Possibly from the aforementioned reasons regarding peace and order in Southern Philippines, the *P. falciparum* in Mindanao has been studied least for drug resistance. Furthermore, chloroquine has been used to treat *P. vivax* malaria in the Philippines since the late 1940s and remains as the interim first-line treatment. It has not been explored whether decades of chloroquine pressure has compromised its usefulness against *P. vivax* in Mindanao.

Hence, this study focused on three malaria endemic provinces in Mindanao to address these gaps in malaria research and to provide evidence-based data that could be used by these provinces and the rest of the country for developing and improving policies towards eliminating malaria in the Philippines by 2020.

2.2. Aims of the study

The main goal of this study was to describe the epidemiology of malaria in three endemic provinces of Sarangani, South Cotabato, and Tawi-Tawi on Mindanao islands, The Philippines and discuss the implications of these findings to malaria elimination. Each specific aim below would be addressed in the succeeding chapters:

1. Estimate the prevalence of *P. falciparum, P. vivax, P. malariae, P. ovale* and the zoonotic *P. knowlesi* in three malaria endemic provinces of Mindanao, The Philippines,
2. Assess malaria transmission intensity in the three endemic provinces of Mindanao using antibody markers of exposure to *P. falciparum* and *P. vivax* infections,
3. Measure the prevalence of drug resistance-associated haplotypes in the chloroquine resistance transporter (*pfcrt*) and multidrug resistance 1 (*pfmdr1*) genes of *P. falciparum* isolates from Mindanao, and examine these genes for novel and previously reported polymorphisms,
4. Determine single-nucleotide polymorphisms in the multi-drug resistance 1 (*pvmdr1*) gene analogue of selected *P. vivax* isolates from Mindanao,
5. Discuss the implications of these findings to eliminating malaria in Mindanao islands, The Philippines

2.3. Plans of the study

Table 2.0 Planned activities during the preparation, field survey, and laboratory assay phases of the study.

Preparation (LSHTM)	Field Survey (Mindanao, Philippines)	Laboratory Assays (LSHTM)
Develop research proposal	Meeting with Philippine collaborators, village officials and rural health unit (RHU) workers	Extracting DNA and sera from blood spots
Apply to Ethics Committees (LSHTM and the Philippines)		Diagnosing Plasmodium by nested PCR
Apply for travel grant to Chadwick Trust (UK)	Planning the field work (training survey staff, mapping the flow of field activities, request for military escort in areas of conflict)	Genotyping of *P. falciparum* isolates
Apply for field work research grant to PCHRD (Philippines)		Genotyping of *P. vivax* isolates
Request RDT sponsorship	Cross-sectional survey: (a) questionnaire survey; (b) field diagnosis of malaria by RDT/microscopy; (c) collection of blood spots on filter paper for molecular and serological assays	Sequencing amplicons from genotyping
Establish collaborations in the Philippines		Determining seroprevalence to *P. falciparum* and *P. vivax* AMA1 and $MSP1_{19}$ antigens by indirect ELISA

The study was divided into four phases: preparation, field survey, laboratory assays (Table 2.0), data analyses and consolidation.

1. The preparation phase was done from September 2009 to April 2010 at the London School of Hygiene and Tropical Medicine (LSHTM). The research proposal was submitted for approval of the researcher's supervisor and Graduate Committee members before permission for involvement of human participants was secured from the LSHTM Ethics Committee and the National Ethics Committee of the Philippines. While these were ongoing, the researcher submitted applications to the Chadwick Trust Travelling Fellowship (London, UK) and the Philippine Council for Health Research and Development (PCHRD) in January 2010. The Chadwick Trust provided £4,000.00 for travel to Sarangani Province in Mindanao, The Philippines in April 2010. The PCHRD funding amounting to £13,875.00 was approved in May 2012 with the University of the Philippines Los Baños as the implementing agency. The PCHRD funding was used to finance the fieldwork in the provinces of Tawi-Tawi and South Cotabato headed by UPLB collaborators. The researcher also sought sponsorships for 1,000 pieces of

RDT FalciVax™ from the Tulip Group of Companies (India) and from Stanbio Laboratory (USA) for the two pieces of STAT-Site®M^{Hgb} Haemoglobin Photometer and 400 pieces of STAT-Site®M^{Hgb} Test Cards to be used in Sarangani Province. Collaborations were established with National and Provincial Malaria Control Programs in the Philippines during this period.

2. The first fieldwork was conducted in Sarangani Province from June to August 2010. However, the researcher has to go on Interruption of Studies (IoS) from the LSHTM from October 2011 to May 2012 to secure additional funding for PhD studies[13]. Upon return to LSHTM, arrangements were made to proceed with the fieldwork in Tawi-Tawi in August to October 2012 and in South Cotabato in February 2013. The timing of fieldwork in these provinces was also dependent on the PCHRD funding, which was only approved and released in May 2012. The researcher went to Sarangani to meet with local collaborators, local officials, tribal leaders, and rural health workers to plan the fieldwork. Field staffs were also trained to conduct the questionnaire survey, to use the field equipment and diagnostics, and to collect blood spots on filter paper. The UPLB collaborators headed the fieldwork in Tawi-Tawi and South Cotabato. The same procedure ensued as the fieldwork in Sarangani, except for requesting military escort in Tawi-Tawi to ensure the safety of field staffs when going around study sites in the different islands. The fieldwork in South Cotabato was timed in February 2013 because the walking trails going to upland communities were non-passable because of heavy rains in the previous months.

3. The accomplished questionnaire surveys and blood spots on filter paper from all study provinces were sent to London. All molecular assays were conducted in the

[13] The researcher was initially funded by the Ford Foundation International Fellowships Program (New York, USA) for two years from Sept 2009-Sept 2011 to pursue MPhil/PhD in Infectious and Tropical Diseases. At the time the IFP no longer supported three-year studies for Philippine fellows. Because of the desire of the researcher to upgrade to PhD status, M. Dacuma went on Interruption of Studies from October 2011-May 2012 to secure further funding. The University of the Philippines System Doctoral Studies Fund approved the funding exactly on the researcher's birthday in Nov. 2011 but since M. Dacuma was already given teaching duties at the UPLB for November 2011 to May 2012, she returned to LSHTM in June 2012 and successfully upgraded in August 2012.

Sutherland Laboratory while all serological assays were conducted in the Drakeley Laboratory at the LSHTM. The nested PCR amplicons from genotyping of Mindanao isolates were sequenced through the centralised sequencing service provided at the LSHTM. The researcher sent the results of nested PCR diagnoses to the respective Provincial Health Offices of Sarangani, South Cotabato and Tawi-Tawi to inform them who among the participants in the study were found infected with malaria. Partial results of the study were also presented as poster at the American Society of Tropical Medicine and Hygiene (ASTMH) Annual Meetings in 2010, 2011 and 2013.

4. Data analyses and consolidation were conducted at the LSHTM. Results of these were presented in the succeeding chapters and were readied for publication.

Chapter 3. Subpatent *Plasmodium* infections: challenge to malaria elimination in Sarangani, South Cotabato and Tawi-Tawi provinces in Mindanao islands, The Philippines

3.1. Background

The Philippines is a country in the Western Pacific that successfully reduced more than 75% of its malaria incidence from 2001 to 2012 (World Health Organization, 2013). It has scaled up vector control towards universal coverage of long-lasting insecticide-treated nets (LLITN) and implemented IRS where necessary, provided prompt and better diagnostics in endemic *barangays*, and adopted ACT policy in 2009 as the cornerstone of *P. falciparum* treatment (Department of Health Philippines, 2011, Liu et al., 2013). As a country with sustained reduction of malaria cases, the Philippines could base its strategies on local conditions when moving from malaria control to elimination agenda (Smith Gueye et al., 2013). The reason for this is rather straightforward. Policies designed for controlling malaria to a level where it will no longer pose a public health threat differ from those targeting malaria elimination, which aim for complete interruption of indigenous malaria transmission (Carter et al., 2000, Moonen et al., 2010).

In low endemic settings, heterogeneous transmission becomes clustered into geographic foci (Bousema et al., 2010a). A focus of malaria transmission is defined by the WHO as a particular spot in a current or recently malarious area having epidemiologic factors to sustain transmission (World Health Organization, 2012a). Mosquito vectors in a malaria focus are capable of sustaining the basic reproductive ratio R_o at a level above one, which is necessary to maintain transmission (Carter et al., 2000). Within each geographic focus are smaller foci or "hotspots", which refer to groups of households with higher risk of malaria transmission (Bousema et al., 2012). Hotspots have been recognised as obstacles to malaria elimination because these can sustain residual malaria transmission (Moonen et al., 2010, Bousema et al., 2012). The infectious human reservoirs in hotspots are often asymptomatic carriers (Bousema et al., 2004, Harris et al., 2010) who harbor the asexual stages of *Plasmodium* but do not manifest the clinical signs of the disease (Kern et al., 2011). These people do not seek treatment (Ogutu et al., 2010, Ganguly et al., 2013) and are capable of infecting mosquitoes to sustain transmission (Shekalaghe et al., 2007, Ouedraogo et al., 2009,

Karl et al., 2011). A robust surveillance system needs to capture and target this hidden burden of malaria if transmission has to be terminated (Sturrock et al., 2013).

In many countries including the Philippines, microscopy remains the operational standard for malaria diagnosis in control and elimination (malERA Consultative Group on Diagnoses and Diagnostics, 2011, Smith Gueye et al., 2013). Microscopy could effectively diagnose *Plasmodium* in medium and high transmission areas (Okell et al., 2012). In low transmission settings microscopy is more likely to underestimate malaria burden and can miss up to 50 percent of subpatent infection compared to polymerase chain reaction (PCR) (Males et al., 2008, Okell et al., 2009, Steenkeste et al., 2010, Mosha et al., 2013). This is because the chance of detecting *Plasmodium* is reduced when the level of parasitaemia is below an average of 25 parasites per microliter (µL) blood (Snounou and Singh, 2002). Skilled microscopist can diagnose as low as five to ten parasites per microliter blood but in field settings the more realistic threshold for microscopy is 100 parasites per microliter blood (World Health Organization, 1988, World Health Organization, 2000).

The development of malaria rapid diagnostic tests (RDT) has improved point-of-care diagnosis of clinical malaria especially in areas with limited or no access to quality microscopy (World Health Organization, 2009). A RDT is an immunochromatographic flow device with impregnated monoclonal antibodies that capture *Plasmodium* antigen from whole blood and provides results in 15 minutes (World Health Organization, 2000). When the tested antigen is present in sufficient concentration, color change is observed on the adsorbent nitrocellulose strip (WHO Regional Office for the Western Pacific and Tropical Disease Research, 2006). Compared to microscopy, the detection threshold of RDT is 40 to 100 parasites per microliter blood (World Health Organization, 2000). The histidine-rich protein 2 (HRP2) is used in many RDTs with sensitivity and specificity greater than 90% for *P. falciparum* diagnosis (Forney et al., 2001, Ly et al., 2010, Elahi et al., 2013). The HRP2 is a 60-105 kDa water-soluble protein expressed by asexual stages of *P. falciparum,* released from infected erythrocytes and circulates in the plasma of infected individuals (Howard et al., 1986). The sensitivity of HRP2-based RDT is influenced by parasite density (Bell et al., 2005),

to some extent by *hrp2* sequence diversity (Lee et al., 2006a, Baker et al., 2010), deletions of *hrp2* genes in *P. falciparum* (Gamboa et al., 2010, Koita et al., 2012), and persistence of HRP2 in the blood after clearance of infection (Mayxay et al., 2001, Iqbal et al., 2004).

The parasite-specific lactate dehydrogenase (pLDH) is an enzyme involved in the glycolytic pathway and this is used for diagnosis of genus *Plasmodium* (pan-pLDH), *P. falciparum* (Pf-pLDH) and *P. vivax* (Pv-pLDH) infections (Fogg et al., 2008, Ashley et al., 2009). The sensitivity of many pLDH-based RDTs is greater than 90% when parasite density is above 100 parasites per microliter blood (Fogg et al., 2008, Ashley et al., 2009, Heutmekers et al., 2012). Unlike the HRP2, the low genetic diversity in pLDH is not associated with differences in sensitivity for *P. falciparum*, *P. vivax* and *P. malariae* diagnosis but may be a contributing factor to poor diagnosis of *P. ovale* spp. and *P. knowlesi* (Talman et al., 2007, Barber et al., 2013). The third protein targeted by RDTs is the *Plasmodium* aldolase, an enzyme of the glycolytic pathway produced as species-specific in *P. falciparum* and *P. vivax* (Murray et al., 2003). However, aldolase-based RDTs have lower sensitivity for *Plasmodium s*pecies compared to HRP2 and pLDH (Harris et al., 2010) despite genetic variability in aldolase gene of different species (Lee et al., 2006b). In an elimination setting it is crucial to detect all malaria infections and the choice of field diagnostics will impact the success of this programme (World Health Organization, 2007).

Each of the 53 endemic provinces will have different challenges for elimination because of heterogeneity of malaria across provinces and islands (Department of Health Philippines, 2011, Philippines National Malaria Program, 2014a). Endemicity of malaria in Philippine provinces can be stable, unstable or epidemic-prone (Department of Health Philippines, 2011) but very little is known about malaria in Mindanao Islands. Understanding the local epidemiology can circumspectly tailor strategies to local situation (Harris et al., 2010). In the Philippines *P. falciparum* and *P. vivax* account for 69% and 31% of malaria cases, respectively (World Health Organization, 2013). There are no estimates on the prevalence of *P. malariae*, *P. ovale* spp. or *P. knowlesi*. Annual malaria prevalence reports can actually underestimate the true prevalence of malaria

in provinces where malaria is successfully reduced. This is because in low transmission settings most of the infected people are asymptomatic and *P. vivax* densities in the population are lower compared to *P. falciparum* (Koepfli et al., 2011, Sturrock et al., 2013). In addition, it is not known whether human infections with *P. knowlesi* are present in Mindanao. These have been reported in the Palawan Islands and the natural host, the long-tailed macaque *Macaca fascicularis* is found throughout the Philippine archipelago (Luchavez et al., 2008). Understanding the local epidemiology will largely contribute to improving local strategies for malaria elimination. Hence, this study aimed to (1) determine the *Plasmodium* species in the provinces of Sarangani, South Cotabato and Tawi-Tawi in Mindanao islands, The Philippines; (2) to estimate the point prevalence of these *Plasmodium* species by PCR; (3) and to evaluate the performance of field microscopy and commercial antigen detection RDT FalciVax™ using PCR as reference standard in diagnosing malaria infection.

3.2. Methods

Ethics Approval

The London School of Hygiene & Tropical Medicine Ethics Committee (Reference No. 5712) (Appendix 1) and the National Ethics Committee of the Philippines (Appendix 2) have approved the involvement of human participants in this study in 2010. Adults signed prior informed consent (Appendix 3) before participating in the survey. A parent or guardian signed for minors who were less than 18 years at the time of the survey. Minors who were seven to less than 18 years of age were requested to sign an assent form in addition to their parent or guardian's consent. Participants were provided with leaflets with information about the study and their rights as participants.

Study Sites

Three malaria endemic provinces in the Mindanao islands were selected for this study: the Sarangani Province and South Cotabato Province in Mindanao mainland and the Tawi-Tawi Province. These provinces were selected because there was a need for baseline epidemiological data to assist these provinces in evaluating existing malaria control, diagnosis and interventions. These provinces have vulnerable population, which could be as indigenous tribal communities or displaced population from local armed conflicts living in geographically isolated and disadvantaged areas (GIDA) that would likely impact malaria control and elimination. Most importantly, there were no previous researches on circulating malaria parasites, anti-malaria drug resistance and malaria transmission in these provinces, which could have otherwise contributed to planning malaria elimination. The unstable peace-and-order situation arising from local armed conflicts in Southern Mindanao likely deterred researches into the region.

Figure 3.1. Map showing coastal Sarangani Province, which is separated from southern part South Cotabato Province by mountain ranges. The surveyed municipalities in Sarangani Province were (1) Kiamba, (2) Maasim, and (3) Glan while the surveyed municipalities in South Cotabato Province were (4) Lake Sebu and (5) T'boli. Inset is the map of the Philippines and the location of the two provinces in mainland Mindanao.

Sarangani Province is along the southeastern coast of Mindanao between latitude 5° 33’ 41” to 6° 32’ 4” North and between longitude 124° 21’ 39.6” to 125° 35’ 11” East (Figure 3.1). It faces Indonesia across the Celebes Sea and shares geographic border with malaria endemic provinces of South Cotabato on the North, Davao del Sur on the East and Sultan Kudarat on the West. Sarangani Province has an estimated total land area of 1,539 square miles. It has seven municipalities and 140 villages or *barangays* in Tagalog, which are considered the smallest political units in the country. Its total population was 498,904 in 2010 and is among the poorest municipalities in the Philippines (Province of Sarangani, 2010). Sarangani Province has type IV climate characterised by evenly distributed rainfall throughout the year (PAGASA, 2004) that would likely contribute to year-round transmission of malaria. It has scaled up its malaria control with support from the Global Fund through the Pilipinas Shell Foundation that led to 89% reduction in reported malaria cases from 2005 to 2010 (Figure 3.2).

South Cotabato Provinces is a land-locked province in Southern Mindanao. It is located at latitude 6°15’ North and longitude 125° 00’ East (Figure 3.1) with a total land area of 1,430.89 square miles. This province shares geographic borders with Sultan Kudarat on the North and West, and the Sarangani Province on the East and South. South Cotabato Province has ten municipalities and the Koronadal City. It has 109 *barangays,* 21 of which are classified as classified as geographically isolated and disadvantaged areas (GIDA) (South Cotabato Provincial Health Office, 2008). As in Sarangani Province rainfall is evenly distributed throughout the year in South Cotabato contributing to year-round malaria transmission. The estimated population in South Cotabato was 1,381,938 in 2012. It has achieved 100% reduction in malaria prevalence from 2001 to 2010 (Figure 3.2) and has not reported any indigenous malaria case from 2010 to 2012 (Philippines National Malaria Program, 2013).

Although geographically adjacent, municipalities of Sarangani Province sharing borders at the southern part of South Cotabato Province are separated by mountain ranges extending to Sultan Kudarat. Upland communities along the borders of each province are not in direct contact with one another and the only route to reach either province is through the common portal through the General Santos City (Figure 3.1). The endemicity of malaria appeared to be different between the two provinces (Figure 3.2). Sarangani Province was classified as having stable malaria transmission while South Cotabato has unstable malaria (Philippines National Malaria Program, 2014f).

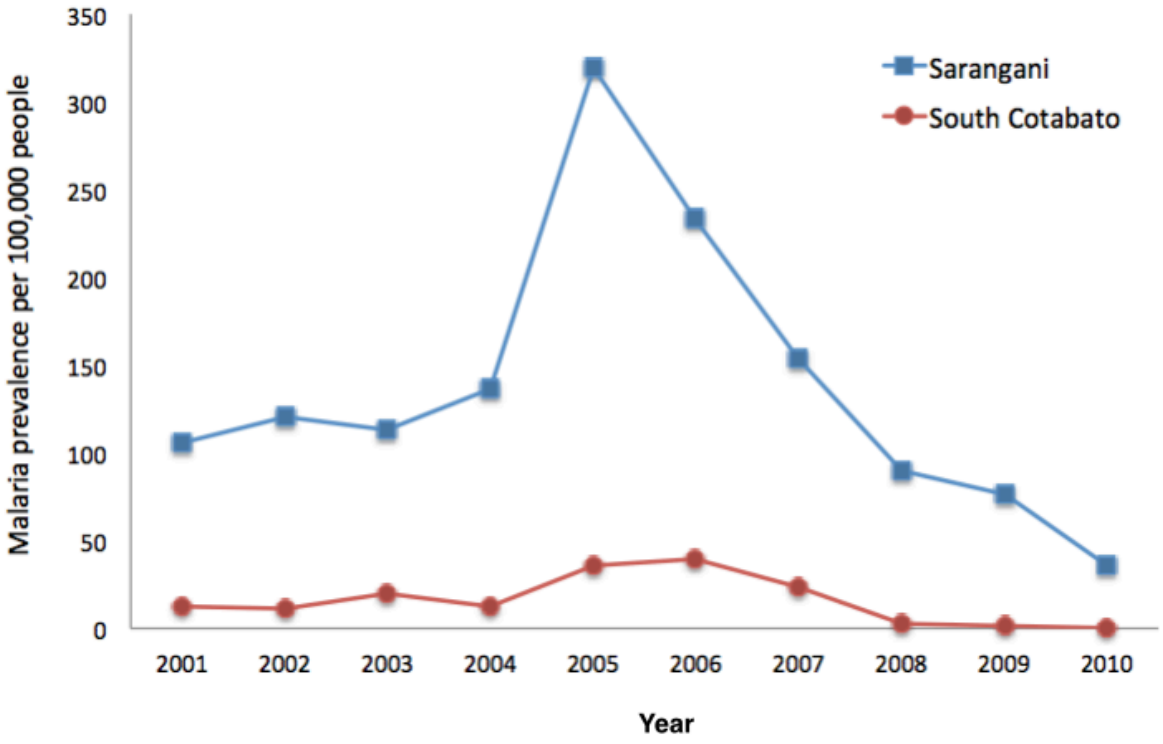


Figure 3.2. Annual prevalence of malaria in Sarangani Province and South Cotabato Province from 2001 to 2010 (Philippines National Malaria Program, 2013).

The Tawi-Tawi Province is among the five provinces of the Autonomous Region in Muslim Mindanao (ARMM). It is at the southernmost tip of the Philippines at latitude 5°10' North and longitude 120° 15' East and shares sea borders with Sabah, Malaysia and North Kalimantan Province, Indonesia. It consists of 307 islands and islets clustered into three islands groups - the Tawi-Tawi Island group, the Tawi-Tawi de Cagayan Island group and the Turtle Islands - with a total land area of 1,323.02 square miles (Figure 3.3). It has 11 municipalities and 203 *barangays* (Abigan, 1998). Like the rest of the Mindanao islands, Tawi-Tawi has evenly distributed rainfall throughout the year (PAGASA, 2004). In 2013 malaria in Tawi-Tawi was still classified as stable high with more than 1,000 cases per year (Philippines National Malaria Program, 2014a). There was no available source for malaria prevalence in Tawi-Tawi from 2000 to 2010 similar to that obtained for Sarangani Province and South Cotabato Province.

These three provinces are home to many indigneous tribes or *lumads*, who live along coastal areas or geographically isolated and difficult to access areas (GIDA) in Mindanao. Through history, these provinces underwent political instabilities, local armed conflicts of the MNLF and MILF against the Philippine government, and civilian unrests that drove population movement and rendered many families below the poverty line (Bertrand, 2000, Ringuet, 2002, Vellema et al., 2011).

Figure 3.3. Map of Tawi-Tawi Province showing the surveyed municipalities (1) Bongao, (2) Panglima Sugala, (3) Languyan and (4) Tandubas. Inset is a map of the Philippines showing the location of Tawi-Tawi Province relative to other islands in the archipelago.

Study Design

This study used cross-sectional survey design in selected municipalities of Sarangani, South Cotabato and Tawi-Tawi provinces. Using one-sample comparison of proportion to a hypothesized value, it was assumed that if the prevalence of malaria was 0.001 or 1 in 1,000 population by microscopy then a minimum of 263 participants per municipality would allow detection of a PCR prevalence of 0.01 or 1 malaria infected person per 100 persons surveyed at a statistical power of 0.80.

Survey Questionnaire

Two native speakers independently translated the English version of the questionnaires (Appendix 2) into *Tagalog* and *Cebuano* dialects. The researcher and the Philippine collaborators reviewed the translations. Before each survey, members of the field team were briefed and sensitized on how to ask the questions from participants to ensure that the intent of each question was satisfied.

Survey Profile

Cross-sectional surveys were conducted in three municipalities of Sarangani Province in 2010, in four island municipalities of Tawi-Tawi Province in 2012 and in two municipalities of South Cotabato Province in 2013. The selected study sites in Sarangani Province and Tawi-Tawi Province were said to be among major contributors to malaria cases in the province. In South Cotabato, malaria cases were zero by microscopy since 2010 but there were unconfirmed reports of malaria in remote tribal communities. Hence, the municipalities of T'boli and Lake Sebu were selected as survey sites. As mentioned in Chapter 1 these three provinces have Type IV climate characterised by evenly distributed rainfall throughout the year. The surveys in Sarangani Province were conducted from June 2010 to August 2010 and in Tawi-Tawi Province from August 2012 to October 2012. The surveys in South Cotabato Province were conducted in February 2013 because the roads to remote upland communities were inaccessible during the rainy season. The timing of the surveys was also

were inaccessible during the rainy season. The timing of the surveys was also influenced by coordination with local officials, availability of local collaborators to guide the field team to study sites, availability of RDT and the status of peace-and-order in the study sites especially in Tawi-Tawi Provinces. Participants of the cross-sectional surveys were selected based on the following inclusion criteria: (1) should be one year old and above at the time of the survey, (2) resided in the municipality in the previous six months from the time of the survey, and (3) has signed the prior informed consent form. Persons with chronic diseases not related to malaria and were taking medications for these chronic diseases were excluded from the survey.

Consenting participants were gathered at a commonplace in the village or *barangay* (Figure 3.4), which was often the Rural Health Centre. Demographic and socio-cultural information was collected from each participant (Appendix 4). This was followed by collection of finger-prick blood for field diagnosis of malaria. The rapid diagnostic test (RDT) FalciVax™ (Zephyr Biochemicals, India) was used to diagnose *P. falciparum* and *P. vivax* infection among participants who attended the cross-sectional surveys in Sarangani Province in 2010 and in South Cotabato Province in 2013. The RDT was processed and interpreted following manufacturer's instructions (Figure 3.5). There was a delay in the purchase of the RDT from manufacturer such that microscopy was used to diagnose malaria among participants in the cross-sectional surveys in Tawi-Tawi Province in 2012. Two registered municipal hospital medical technologists in Tawi-Tawi Province independently examined the blood films collected from participants for presence of malaria parasites. In the event of discordance between the two microscopists, the slide would be submitted to the provincial microscopy validator. Those diagnosed with malaria by RDT or microcopy were referred to the Rural Health Centres for treatment following national guidelines. Finger-prick blood as spots on 3MM Whatman® chromatography paper were also collected from consenting participants. These were air-dried and stored with desiccant at ambient temperature before transport to the LSHTM in the United Kingdom.

Figure 3.4. Consenting participants gathered at a commonplace in one of the surveyed *barangays* in the municipality of Kiamba in Sarangani Province.

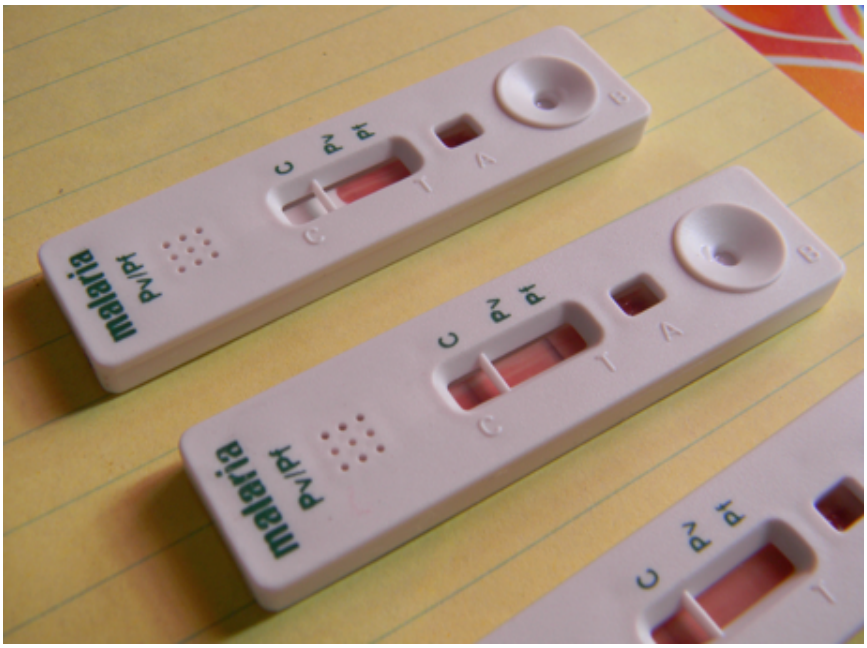


Figure 3.5. The rapid diagnostic test FalciVax™ used for field diagnosis of *P. falciparum* and *P. vivax* malaria in Sarangani Province and South Cotabato Province.

DNA Extraction

At the LSHTM dried blood spots on filter paper were stored at 4°C prior to molecular analyses. DNA from the blood spots was extracted by boiling in Chelex® 100 (Sigma-Aldrich) as described by Plowe et al. (1995). In this study, 6mm diameter of each participant's dried blood spot on filter paper was cut using a Harris Uni-Core puncher and placed into a 96 deep well plate. Ten negative controls or blanks were randomly placed in the plate during DNA extraction. The samples were incubated overnight at room temperature after adding 1 mL of 0.5% saponin in 1X Phosphate Buffered Saline (PBS) solution.

The next day, the samples were centrifuged for 2 min at 4,000 rpm using Eppendorf Centrifuge 5810R (Eppendorf, United Kingdom). The PBS containing saponin, cell lysate, and blood sera from each well was transferred into a sterile 96 deep well plate and stored at -40°C prior to serological assay (see Chapter 6). Thereafter, 1000 µL of 1X PBS was added to the 96-deep well plate containing the punched filter papers to remove traces of saponin by centrifuging the plate for 2 min at 4,000 rpm. The PBS from each well was removed before adding 150 µL of 6% Chelex® 100 (Sigma-Aldrich). The plate was then heat-sealed with thermo sealing foil before placing it in boiling water bath. After 45 minutes the 96-well plate was removed from the boiling water bath, allowed to cool down before centrifuging for 5 min at 4,000 rpm to spin down the Chelex. Approximately 100µL supernatant containing the sample DNA was transferred from the 96 wells deep well plate to a sterile PCR plate, which was stored at -20°C prior to molecular analyses.

Diagnosis of *Plasmodium* infections by PCR

DNA from all participants was screened by PCR for presence of *Plasmodium* species using the protocol of Snounou et al. (1993) and Singh et al. (Singh et al., 2004) regardless of RDT and microscopy results. The primers used, which target the 18S small subunit of ribosomal RNA (ssrRNA) gene of *Plasmodium*, were presented in Table 3.1. For the genus amplification, 5 µL DNA were added to PCR mix containing 1X NH4 buffer (Bioline), 2mM $MgCl_2$ (Bioline), 62.5 µM each deoxyribonucleotide (dNTP) (Bioline), 250 nM rPLU6 forward primer, 250nM rPLU5new reverse primer, and 1 unit (U) BIOTAQ™ DNA polymerase (Bioline). The final volume for each reaction was adjusted to 20 µL. One microliter of the primary PCR product was used as template to each of the five different nested PCRs designed for speciation. The nested PCR mix has the same components and final volume as the primary PCR, except for the template and the primer pairs used. Negative controls from DNA extraction and PCR no template controls (NTC) were used per PCR assay. The LSHTM Malaria Reference Laboratory (MRL) provided the DNA standards for *P. vivax, P. malariae* and *P. ovale* spp. Laboratory grown 3D7 isolate was used as reference standard for *P. falciparum* while the *P. knowlesi* DNA was obtained from *P. knowlesi*-infected monkey blood from Guy's Hospital, Kings College in London. The positive controls were diluted to 1:100 from the stock solutions.

The reactions were carried out in G-storm GS2 thermal cycler (Labtech International Ltd., United Kingdom). The cycling parameters for the primary PCR included 5 min denaturation at 95°C followed by 25 cycles, each of annealing at 58°C for 2 min, extension at 72°C for 2 min and denaturation at 94°C for 1 min. These were followed by final annealing at 58°C for 5 min and final extension at 72°C for 5 min. The nested PCR amplification of *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale* spp. was carried out using the same cycling parameters as the primary PCR, except that number of cycles after the initial denaturation was increased to 30 cycles (Snounou et al., 1993). For *P. knowlesi*-specific nested PCR amplification, the annealing temperature was changed to 60°C (Singh et al., 2004).

Table 3.1. Primers targeting the 18S small subunit of the ribosomal RNA gene of *Plasmodium*.

Primer[1]	Primer sequence (5'-3')	Size (bp)	Reference
Primary or genus-specific PCR			
rPLU6	TTAAAATTGTTGCAGTTAAAACG	1200	(Snounou et al., 1993)
rPLU5new[2]	CYTGTTGTTGCCTTAAACTTC		
Nested or species-specific PCR			
a. *P. falciparum*			
rFAL1	TTAAACTGGTTTGGGAAAACCAAATATATT	205	(Snounou et al., 1993)
rFAL2	ACACAATGAACTCAATCATGACTACCCGTC		
b. *P. vivax*			
rV1V1	CGCTTCTAGCTTAATCCACATAACTGATAC	120	(Snounou et al., 1993)
rV1V2	ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA		
c. *P. malariae*			
rMAL1	ATAACATAGTTGTACGTTAAGAATAACCGC	144	(Snounou et al., 1993)
rMAL2	AAAATTCCCATGCATAAAAAATTATACAAA		
d. *P. ovale* (classic and variant)			
PadPo	CTGTTCTTTGCATTCCTTATGC	843	(Padley et al., 2003)
rOVA2v	GGAAAAGGACACTATAATGTATCCTAATA		(Calderaro et al., 2007)
e. *P. knowlesi*			
Pmk8	GTTAGCGAGAGCCACAAAAAAGCGAAT	153	(Singh et al., 2004)
Pmkr9	ACTCAAAGTAACAAAATCTTCCGTA		

[1]The primers are arranged as forward primer followed by reverse primer.
[2]The LSHTM Malaria Reference Laboratory (MRL) modified a single base in the original rPLU5 primer of Snounou (1993).

Use of *pfmdr1* and *pvmdr1* genes for identifying more samples with *P. falciparum* and *P. vivax* infections

PCR protocols to determine molecular markers of drug resistance were also used to identify more *P. falciparum* and *P. vivax* positive samples in all provinces surveyed. These PCR assays were described in Chapters 4 and 5 respectively. PCR primers targeting the *P. falciparum* multidrug resistance gene 1 (*pfmdr1*) codons 86 and 184 (Humphreys et al., 2007) was found in the Sutherland Laboratory to be very specific for

P. falciparum (unpublished data) and this was used to identify more *P. falciparum* positives after using the Snounou primers for *P. falciparum.* The limit of detection of this PCR assay was not determined using control DNA of *P. falciparum* from blood with known parasite density and extracted from filter paper using the Chelex method. Samples positive for *P. falciparum* in the Snounou PCR or the *pfmdr1* codons 86 and 184 genotyping PCR or both were recorded with *P. falciparum* infection.

The PCR assay using hemi-nested primers targeting *pvmdr1* codon 91 was found to be specific for *P. vivax* (Figure 3.6). Nested primers have been previously tested for this codon but were not successful in optimising the PCR assay. Its limit of sensitivity was not determined because there was no control *P. vivax*-infected blood with known parasite density. This PCR assay was used to screen for more *P. vivax* positive samples in the three provinces surveyed. Samples that were positive using Snounou primers or genotyping primers or both were recorded positive for that particular *Plasmodium* species.

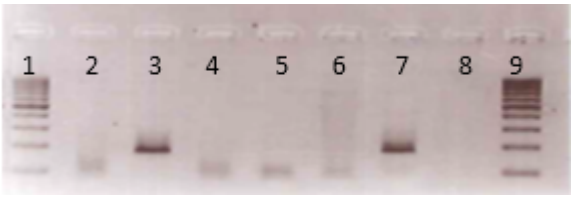


Figure 3.6. Testing the nested PCR primers targeting 184 base pair fragment around *pvmdr1* codon 91 against other *Plasmodium* species at 62°C annealing temperature. Lanes 1 & 9: DNA 100 bp ladder, lane 2: *P. falciparum* 3D7 laboratory grown strain, *P. vivax* clinical isolate MRL 11/0890, lane 4: *P. malariae* clinical isolate MRL, lane 5: *P. knowlesi* H strain MR4-456G genomic DNA, lane 6: *P. ovale curtisi* strain MRL 10/1058; lane 7: *P. vivax* sample TKE 022 (Sarangani Province, Philippines) diagnosed by RDT (FalciVax™) and by nested PCR (Snounou et al., 1993) and lane 8: no template control (NTC).

Electrophoresis and viewing of PCR amplicons

Amplicons from species-specific PCR and molecular marker PCR assays were run in 2.5% and 2.0% agarose gel electrophoresis, respectively. The agarose gel contained 0.75µg per mL ethidium bromide (Sigma-Aldrich) and eluted at 0.5X Tris-Borate-EDTA (TBE) buffer. The PCR products were allowed to run in gel electrophoresis for 40 minutes at 120V after which the gel was viewed under a UV-transilluminator. The PCR assay was valid if the positive controls were amplified and there was no evidence of contamination in the negative controls. A PCR assay was repeated when there was evidence of contamination on the PCR negative controls.

PCR amplicons sequencing and alignment

The amplicons obtained from the *pfmdr1* and *pvmdr1* gene assays were sequenced using the BigDye® Terminator V3.1 cycle sequencing kits (Applied Biosystems) and analysed on an ABI 3730 sequencer (Applied Biosystems). The resulting sequence chromatograms were viewed using Geneious® version 7.0 (Biomatters, New Zealand) and Chromas version 1.61 (Technelysium Pty Ltd., Queensland). The sequences were aligned to sequences in the National Center for Biotechnology Information (NCBI) database using the nucleotide Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/) for confirmation of sequence identity of *P. falciparum* and *P. vivax*.

Data management and statistical analyses

Data were encoded in Microsoft Excel (Microsoft Corp., USA) and checked for errors in data entry before analysing in Stata version 13 (Stata Corp., College Station, Texas). Results of microscopy or RDT were matched with results of speciation and genotyping PCR and serological screening. A sample has malaria infection if it has positive result by PCR regardless of the results of microscopy or rapid diagnostic test.

The point prevalence of a *Plasmodium* species per province was calculated as follows:

$$\text{Point prevalence (\%)} = \frac{\text{n samples that were positive using the test}}{\text{Total number (N) of samples tested}} \times 100$$

Data from questionnaire survey were summarised. Descriptive statistics for continuous variables were presented as mean values and standard deviation while categorical variables were presented as percentages.

Performance of RDT and microscopy compared to PCR for diagnosis of malaria

In most researches the diagnostic performance of a malaria index test would be evaluated against microscopy. In this study parasite density was expected to be low and possibly at the limit of detection of microscopy such that PCR would be more sensitive in diagnosing *Plasmodium* infections (Okell et al., 2009). Thus, the diagnostic performance in terms of sensitivity and specificity of field diagnostics RDT and microscopy were compared to PCR using the diagnostic method developed by Seed and Tobias (2001). The 'diagti' command in Stata version 13 would calculate the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of RDT or microscopy compared to PCR, which was used as the reference standard. Sensitivity was the proportion of samples positive for malaria by PCR that were positive for malaria using RDT or microscopy. Specificity was the proportion of samples negative for malaria by PCR that were negative for malaria using RDT or microscopy. The positive predictive value is the proportion of malaria positives using RDT or microscopy that were malaria positives by PCR. The negative predictive value was the proportion of negatives using RDT or microscopy that were negative by PCR.

3.3. Results

Description of the study population

A. Sarangani Province

The provinces of Sarangani, South Cotabato and Tawi-Tawi were chosen as study sites because of need for evidence-based epidemiological data in these areas. Sarangani Province is separated from South Cotabato Province by stretch of mountain ranges reaching up to more than 400 meters above sea level such that communities along the borders have no direct contact. Sarangani Province differed from South Cotabato Province in malaria endemicity (Figure 3.2) suggesting that each needed different control and elimination strategies. Three municipalities were surveyed in Sarangani Province in 2010. These were the municipalities of Glan, Kiamba and Maasim, which were major contributors to malaria cases in the province (Provincial Health Office of Sarangani, personal communication). A total of 939 people were surveyed, which constituted 3.0% of the total population in these municipalities. Two hundred ninety four (31%) participants were from the Municipality of Glan, 337 (36%) were from the Municipality of Kiamba and 308 (33%) were from the Municipality of Maasim. Majority (69%) of the participants lived in communities with poor access. The survey team has to travel through old logging roads or riverbanks to reach these communities (Figure 3.7). During the survey 44.5% of the participants were children (<15 years) and 83.3% were members of indigenous tribes. About 40.8% lived in houses made of bamboo and 92.4% claimed that they slept under an insecticide-treated net (ITN) at night. Their main source of livelihood was farming and about 81% lived more than 500 meters away from the forest, which was defined in this study as an area covered with trees or underbrush and has not been cleared for agricultural use. About 12% (508) of participants aged 7 years and above observed a monkey or monkeys within 500 meters from their houses (Table 3.2).

Table 3.2. Characteristics of study population from the three malaria endemic provinces in Mindanao, The Philippines.

	Provinces		
	Sarangani	**South Cotabato**	**Tawi-Tawi**
Participants N	939	600	1,088
Age in years	23.6	18.7	25.0
Mean (SD, range)	(19.6, 1-86)	(14.7, 1-75)	(16.3, 1-100)
<15	418 (44.5)	280 (56.9)	399 (36.8)
≥15	521 (55.5)	212 (43.1)	685 (63.2)
Gender, n (%)			
Female	602 (64.0)	348 (67.7)	560 (51.7)
Male	337 (36.0)	166 (32.3)	523 (48.3)
Type of dwelling n (%)			
Wood	185 (19.7)	303 (60.3)	897 (84.1)
Bamboo	383 (40.8)	142 (29.1)	21 (1.9)
Cement	64 (6.8)	52 (10.6)	149 (14.0)
Any combination	307 (32.7)	0	0
Bed net use n (%)			
Yes, ITN	857 (94.2)	0	779 (72.8)
Yes, non-ITN	22 (2.4)	0	95 (8.9)
Yes, not known if ITN	14 (1.4)	422 (87.2)	57 (5.3)
No	17 (2.0)	62 (12.8)	139 (12.9)
Economic activity n (%)			
None (also included children, students and elderly)	516 (54.9)	324 (65.9)	334 (34.8)
Farmer	206 (21.9)	90 (18.3)	116 (12.1)
Housekeeper	135 (14.4)	54 (11.0)	219 (22.8)
Fisherman	26 (2.8)	1 (0.2)	83 (8.7)
Soldier	0	0	191 (19.9)
Others	56 (6.0)	23 (4.7)	17 (1.8)
Ethnic Group n (%)			
T'boli	342 (36.4)	509 (98.1)	0
B'laan	309 (32.9)	0	0
Maguindanao	98 (10.4)	10 (1.9)	0
Tausug	34 (3.6)	0	461 (42.4)
Samal	11 (1.2)	0	416 (38.2)
Other minorities	48 (5.1)	0	1 (0.1)
Non-minorities	92 (9.8)	0	210 (19.3)
Distance of house from the forest			
Within 500 meters or less	170 (18.8)	431 (88.5)	888 (82.6)
Greater than 500 meters	734 (81.2)	56 (11.5)	187 (17.4)
Spends the night within 500 meters from the forest or in the forest n (%)			
Yes	169 (18.6)	429 (88.1)	985 (91.7)
No	741 (81.4)	58 (11.9)	89 (8.3)

A

B

Figure 3.7. The highland remote community of B'laan tribe in Amsipit village in the municipality of Maasim, Sarangani Province is located at N 05°58.339' E 125°00.074' beyond the stretch of hills shown above (A), and the team has to travel along the bank of the Siguel River (B) to reach the community (Photo credits M Dacuma).

B. South Cotabato Province

South Cotabato Province has no reported malaria cases in its 10 municipalities since 2010. Despite absence of malaria cases by microscopy, there were still suspected cases among remote upland communities of the municipalities of T'boli and Lake Sebu. In 2013 a total of 600 consenting participants were surveyed and this represented 2.4% of the total population in the surveyed villages of the two municipalities. Two hundred seventy (45%) participants were from the municipality of Lake Sebu and 330 (55%) were from the municipality of T'boli. Eighteen percent (108) of the participants did not provide age information. Among those with age data about 56.9% were children (less than 15 years old). Ninety-eight percent of participants were members of the indigenous tribe T'boli. Sixty percent of the participants lived in houses made of wood and 87.2% said they slept under bed net at night but were not certain whether the bed nets were insecticide-treated or not. Since the local government provided nets as part of malaria control activities, it was very likely that these were ITNs. During the survey 88.5% of the participants mentioned that they lived within 500 meters from the forest. Only 4.1% (16) of participants aged 7 years and above observed a monkey or monkeys within 500 meters from their houses (Table 3.2).

C. Tawi-Tawi Province

A total of 1,088 participants from four island municipalities were surveyed in Tawi-Tawi Province in 2012. Sixteen percent of the participants were from the municipality of Bongao, 21% from Laconon, 45% from Languyan and 18% from Panglima Sugala. The survey participants represented 2.9% of the total population among the villages surveyed in these four municipalities. Majority (63.2%) of the participants were adults (15 years and above). As in South Cotabato a large proportion of the participants (84.4%) lived in houses made of wood. Although 72.8% of the participants mentioned that they slept under ITN at night, 8.9% of them slept under non-ITN. Majority (80.6%) of the participants were members of indigenous tribes Tausug and Samal. About 88.5% of them lived within 500 meters or less from the forest (Table 3.2). About 81.7% of

participants aged 7 years and above observed a monkey or monkeys within 500 meters from their houses.

Malaria prevalence estimated by PCR

With the exception of a single clinical case of *P. malariae* in South Cotabato Province, all individuals diagnosed with *P. falciparum* and *P. vivax* infections by PCR did not present with fever during the cross-sectional surveys. Fever was referred to as *lagnat, hilanat* or *kini* in local dialects of Sarangani Province and South Cotabato Province while it was locally referred to as *mapasu in baran* in Tawi-Tawi Province. In this study people with malaria by PCR but did not present or complain with fever at the time of the survey were considered asymptomatic. The overall malaria prevalence by PCR was 3.7% in Sarangani Province, 10% in South Cotabato Province and 4.2% in Tawi-Tawi Province (Table 3.3). The prevalence of *P. falciparum* was higher than that of *P. vivax* in Sarangani Province and Tawi-Tawi Province while it was the opposite in South Cotabato Province. None of the participants has *P. knowlesi* and *P. ovale* spp. infection by PCR. There was one *P. malariae* infection from a person who travelled to South Cotabato from Sultan Kudarat Province before the infection manifested. Because *P. malariae* cases were not common in the Philippines, microscopic blood smear from the patient was screened and validated at the municipal, provincial and regional microscopy validation centres. The slide was also examined and confirmed to have *P. malariae* infection at the LSHTM Malaria Reference Laboratory.

Table 3.3. Point prevalence of *Plasmodium* infections in the study population from three endemic provinces of Mindanao, The Philippines.

Provinces	PCR diagnosis							Total
	Negative	Mixed infection of *P. falciparum* and *P. vivax*	*P. falciparum*	*P. vivax*	*P. malariae*	*P. knowlesi*	*P. ovale* spp.	
Sarangani	904 (96.3%)	0	20 (2.1%)	15 (1.6%)	0	0	0	939 (100%)
South Cotabato	541 (90.2%)	3 (0.5%)	23 (3.8%)	33 (5.5%)	1 (0.2%)	0	0	601 (100%)
Tawi-Tawi	1,042 (95.8%)	0	26 (2.4%)	20 (1.8%)	0	0	0	1,088 (100%)
Total	2,487 (94.6%)	3 (0.1%)	69 (2.6%)	68 (2.6%)	1 (<0.1%)	0	0	2,628 (100%)

Table 3.4. Frequency of malaria diagnosed using RDT FalciVax™ and microscopy compared to diagnosis by PCR.

Province	RDT diagnosis	PCR diagnosis				Total
		Negative	*P. falciparum*	*P. vivax*	Mixed infection of *P. falciparum* and *P. vivax*	
Sarangani	Negative	877 (96.6%)	17 (1.9%)	14 (1.5%)	0	908 (100.0%)
	P. falciparum	7 (70.0%)	3 (30.0%)	0	0	10 (100.0%)
	P. vivax	0	0	1 (100.0%)	0	1 (100.0%)
	Total	884 (96.2%)	20 (2.2%)	15 (1.6%)	0	919 (100.0%)
South Cotabato	Negative	512 (92.1%)	14 (2.5%)	29 (5.2%)	1 (0.2%)	556 (100.0%)
	P. falciparum	1 (100.0%)	0	0	0	1 (100.0%)
	P. vivax	11 (47.8%)	8 (34.8%)	2 (8.7%)	2 (8.7%)	23 (100.0%)
	P. falciparum & *P. vivax*	1 (50.0%)	1 (50.0%)	0	0	2 (100.0%)
		525 (90.2%)	23 (4.0%)	31 (5.3%)	3 (0.5%)	582
	Microscopy					
Tawi-Tawi	Negative	1,042 (95.8%)	26 (2.4%)	20 (1.8%)	0	1,088 (100.0%)
	P. falciparum	0	0	0	0	0
	P. vivax	0	0	0	0	0
	P. falciparum & *P. vivax*	0	0	0	0	0
	Total	1,042 (95.8%)	26 (2.4%)	20 (1.8%)	0	1,088 (100.0%)

Malaria diagnosis using the RDT FalciVax™ and microscopy compared to PCR

Table 3.5. Diagnostic performance of FalciVax™ and microscopy compared to PCR as reference standard.

RDT	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Sarangani Province				
P. falciparum	30.0 (6.7-65.2)	98.1 (97.0-98.9)	15.0 (3.2-37.9)	99.2 (98.4-99.7)
P. vivax	100.0 (2.5-100.0)	98.5 (97.5-99.2)	6.7 (0.2-31.5)	100.0 (99.6-100.0)
South Cotabato				
P. falciparum	33.3 (0.8-90.6)	96.2 (94.3-97.6)	4.4 (0.1-21.9)	99.6 (98.7-99.9)
P. vivax	16.0 (4.5-36.1)	94.6 (92.4-96.3)	11.8 (3.3-27.5)	96.2 (94.2-97.6)
Microscopy				
Tawi-Tawi				
P. falciparum	0	97.6 (96.5-98.4)	0 (0.0-13.2)	100.0 (99.7-100.0)
P. vivax	0	98.1 (97.2-98.9)	0 (0.0-16.8)	100.0 (99.7-100.0)

The RDT has poor sensitivity for diagnosing subpatent *P. falciparum* compared to PCR in Sarangani Province and South Cotabato Province (Table 3.5). Only 30% and 33.3% of *P. falciparum* positives by RDT in Sarangani Province and South Cotabato Province respectively were positive by PCR. The positive predictive values for RDT were very low in both provinces. For instance, only 15% of the *P. falciparum* positive samples by RDT were positive by PCR. In Sarangani Province there was one *P. vivax* infection by RDT, which was also positive for *P. vivax* by PCR and thus, the 100% sensitivity. However, the PPV for *P. vivax* was very low in this province since the RDT failed to diagnose 14 samples with *P. vivax* by PCR (Table 3.4). In South Cotabato, sensitivity and specificity of RDT for *P. vivax* was low compared to PCR. The proportions of subpatent *P. falciparum* and *P. vivax* infections diagnosed by RDT and PCR differed significantly ($p<0.001$) for both species. Microscopy did not find any malaria infection among the

1,088 samples from Tawi-Tawi. However, 4.2% of the participants have malaria by PCR (Table 3.4). In this study, microscopy lacked sensitivity for diagnosing subpatent *P. falciparum* and *P. vivax* infections in Tawi-Tawi Province (Table 3.5). The proportions of subpatent *P. falciparum* and *P. vivax* infection by PCR significantly differed from microscopy at $p<0.001$.

Malaria among children less than 5 years old

In Sarangani Province five children and a child younger than 5 years were diagnosed with *P. falciparum* and *P. vivax* infection by PCR respectively. In South Cotabato two and three children younger than 5 years old were diagnosed with *P. falciparum* and *P. vivax* infection by PCR respectively. PCR diagnosis has been repeated for confirmation among these children. Malaria among children less than 5 years was not observed among participants in Tawi-Tawi Province.

3.4. Discussion

Subpatent malaria in Mindanao

Malaria burden was greatly reduced in the study provinces in Mindanao after scale-up of interventions using ITN, IRS and adoption of ACT-based policy (Department of Health Philippines, 2011, Philippines National Malaria Program, 2013). Locating the remaining infectious reservoirs in the population would promote further reduction of malaria in low endemic areas (Sturrock et al., 2013). This study looked into malaria infections in three endemic provinces of Mindanao. This study did not measure basal body temperature for each participant during the survey. Participants who did not complain of fever during the survey but with *P. falciparum* or *P. vivax* infection by PCR were considered with subpatent malaria infection. There were two participants from Sarangani Province who complained of fever but they were negative for malaria by RDT and PCR. Fever was locally known as *lagnat, hilanat* or *kini* in Sarangani Province

and South Cotabato while it is locally known as *mapasu in baran* in Tawi-Tawi Province.

When the cross-sectional surveys were conducted, Sarangani Province and Tawi-Tawi Province have stable low (<100 cases per year) and stable high (>1000 cases per year) malaria respectively while South Cotabato Province has unstable malaria (Philippines National Malaria Program, 2014f). From this background, it was expected that malaria prevalence by PCR would be higher in Tawi-Tawi Province and Sarangani Province than in South Cotabato Province. Against expectations PCR estimates of malaria was highest in South Cotabato Province (10.0%) compared to Tawi-Tawi Province (4.2%) and Sarangani Province (3.7%). In South Cotabato the participants represented 2.4% (600/25,370) of the total population in three geographically isolated and difficult to access (GIDA) villages of T'boli and Lake Sebu. None of the South Cotabato participants diagnosed with malaria by PCR complained of fever. This suggested that while malaria cases by microscopy were absent in South Cotabato since 2010 (Philippines National Malaria Program, 2013), there were still small pockets of *P. falciparum* and *P. vivax* transmission occurring in remote villages. This low-level transmission possibly maintained the immunity of the tribal communities that limited parasite density among people in the absence of significant exposure (Bustos et al., 1997, Kaneko et al., 2014). People with subpatent malaria have been reported in low transmission and elimination settings (Harris et al., 2010, Okell et al., 2012, Steenkeste et al., 2010). These people would not actively seek diagnosis and treatment and thus, would be parasite reservoirs affecting gains in malaria elimination (Moonen et al., 2010, Sturrock et al., 2013, Starzengruber et al., 2014). With PCR the estimated prevalence of subpatent *P. vivax* (5.5%) was higher that that of *P. falciparum* (3.8%) in South Cotabato. This could be due to activation of hypnozoites among participants with *P. vivax* infection. These findings also suggested that existing control measures effective in reducing *P. falciparum* might have no effect on *P. vivax*.

In accordance with national estimates, *P. falciparum* prevalence was higher than *P. vivax* by PCR in Sarangani Province and Tawi-Tawi Province. As in South Cotabato, none of the participants with malaria by PCR complained of fever during the surveys in

Sarangani Province and Tawi-Tawi Province. The RDT used in Sarangani Province only diagnosed 15% (3/20) and 6.7% (1/15) of *P. falciparum* and *P. vivax* infections by PCR. Meanwhile in Tawi-Tawi Province, none of the *P. falciparum* (2.4%) and *P. vivax* (1.8%) infections by PCR was diagnosed by microscopy. Although the surveyed participants represented 3.0% (939/31,742) of the total population in nine villages surveyed in Sarangani Province and 2.9% (1,088/37,562) of the total population in thirteen villages surveyed in Tawi-Tawi Province, these findings suggested that subpatent malaria infections were present in both provinces. Without a more sensitive diagnostic tool to locate and treat these people, they would constitute important parasite reservoirs that could support local malaria transmission.

Malaria diagnosis using RDT and microscopy

The initial plan was to use RDT for malaria diagnosis in the three provinces but logistics delayed the purchase of RDT kits and thus, microscopy was used for field diagnosis of malaria in Tawi-Tawi Province. Forty-six samples with malaria by PCR were not diagnosed using field microscopy. It was likely that parasite densities in these samples were below the limit of detection of microscopy (Golassa et al., 2013, Mosha et al., 2013). Since microscopy remained as the operational standard for malaria diagnosis in Tawi-Tawi and the rest of the Philippines, these findings suggested that microscopy could miss subpatent malaria (Okell et al., 2009, Rosas-Aguirre et al., 2013) particularly if malaria transmission has reduced. The absence of malaria by microscopy could be erroneously inferred as true absence of malaria in the population (Bell et al., 2005).

The commercial RDT FalciVax™ was used for field diagnosis of *P. falciparum* and *P. vivax* infections in Sarangani Province and South Cotabato Province. This RDT, which could detect *P. falciparum*-specific HRP2 and *P. vivax*-specific LDH antigens in peripheral blood, has high sensitivity and specificity for both species in febrile persons suspected with malaria (Singh et al., 2010, Alam et al., 2011) even in low endemic areas (Meena et al., 2009). Compared to PCR this RDT has poor sensitivity for *P. falciparum* and *P. vivax* among participants with subpatent malaria infections. Low parasite density was a likely factor in its poor performance since the sensitivity of

FalciVax™ would be reduced by 50% when parasite density was less than 100 parasites per microliter blood (Singh et al., 2010). Seven and two participants from Sarangani Province and South Cotabato Province respectively were diagnosed with *P. falciparum* by RDT but were negative for malaria by PCR. This discrepancy between RDT and PCR results could be due to persistent *P. falciparum* HRP2 antigen in the blood of a person after *P. falciparum* infection has cleared out (Mayxay et al., 2001, Iqbal et al., 2004). The RDT also failed to detect 85% (17/20) and 88.5% (23/26) of *P. falciparum* infections by PCR in Sarangani Province and South Cotabato Province respectively. It was very likely that the parasite densities were already at the limit of detection of the RDT (Singh et al., 2010). Another possible reason could be deletion the *pfhrp2* gene or polymorphisms in this gene among local isolates of *P. falciparum* (Gamboa et al., 2010, Akinyi et al., 2013). Further studies would be needed to demonstrate this in Mindanao. The RDT also failed to detect 93.3% (14/15) and 90.9% (30/33) of *P. vivax* infections by PCR in Sarangani Province and South Cotabato Province respectively. In South Cotabato eight samples were diagnosed with *P. vivax* infection by RDT but were positive for *P. falciparum* infection by PCR. This discrepancy in diagnosis could lead to administration of chloroquine and primaquine treatment (Maltha et al., 2010) following national guidelines in the absence of a more sensitive reference standard. It must be noted that RDT diagnosis was based on parasite antigen detection in peripheral blood while PCR diagnosis was based on specific parasite gene amplification. Parasite densities in these subpatent malaria infections would likely be at the limit of detection of both assays.

Implications of malaria in children less than 5 years old

A small number of children who were below five years old were diagnosed with *P. falciparum* and *P. vivax* infections by PCR in Sarangani Province and South Cotabato Province. These children did not complain of fever during the survey. It would have been relevant if axillary temperature was measured from each participant since fever would be a good indicator of malaria (Mutanda et al., 2014). It would be expected that younger children from malaria endemic areas would be febrile with higher parasite densities in blood after exposure to malaria since partial immunity or premunition

would develop with age following subsequent exposure to malaria infections (Missinou et al., 2003, Singh et al., 2014). It was possible that children who participated in this study and found with malaria by PCR have had fever days before the survey but this information was not captured in the questionnaire. It was also possible that at the time of the survey parasite density in their peripheral blood was lower than during febrile stages, which was still detected by PCR. Nevertheless, presence of malaria infection signified prior exposure (Gosling, 2008). Although the number of infected children under five years was small, this suggested recent malaria transmission particularly in South Cotabato Province where malaria cases by microscopy were not reported since 2010. These small pockets of malaria transmission could be identified using serological markers (Bousema et al., 2010c, Cook et al., 2011) as described in Chapter 6. In these low endemic provinces, malaria was most likely persistent and underestimated by microscopy (Bustos et al., 1997). Without active case detection using more sensitive diagnostics comparable to PCR, this burden would remain undetected (Harris et al., 2010, Mosha et al., 2013) and could lead to debilitating conditions such as anaemia and stunted growth in childhood (de Mast et al., 2010).

Implications of imported malaria to control and elimination

It was only in South Cotabato Province where imported *P. malariae* was encountered at the time of survey, but this simple occurrence appeared to have originated from Sultan Kudarat Province, which shared geographic borders with South Cotabato Province. The prevalence of *P. malariae* was very low (<0.1%) in the Philippines and a single case would be significant. While South Cotabato Province endeavored to maintain absence of indigenous malaria by microscopy from 2010, the threat of malaria importation often loomed. South Cotabato Province has no defined policy regarding entry of people from neighboring malaria endemic provinces (South Cotabato Provincial Health Office, 2008), which could hamper elimination when imported malaria became established in the province at a level sufficient to promote transmission (Smith et al., 2013).

The absence of *P. ovale* spp. and *P. knowlesi*

P. ovale spp. and *P. knowlesi* were previously reported in Palawan Islands, The Philippines (Alves et al., 1968, Luchavez et al., 2008) but their extent of geographic distribution and public health threat were not known in the country. Some areas of the Philippines have the potential to transmit *P. knowlesi* as the simian host and mosquito vector are both present (Moyes et al., 2014). However, this zoonotic parasite has not been reported in the Mindanao islands. PCR diagnosis did not find any *P. knowlesi* infection among participants of this study. The simian host of *P. knowlesi*, the long-tailed macaque, was present in all study sites but it was not known whether any of the captive or wild long-tailed macaques have *P. knowlesi* infection. If the monkey population has *P. knowlesi* infection then either this survey was not able to include people at higher risk of having *P. knowlesi* infection or the environment did not support the transmission from simian to human hosts. It was also possible that *P. knowlesi* might not be present among island populations in Mindanao but further studies would be needed to demonstrate this.

3.5. Conclusions

a. Subpatent *P. falciparum* and *P. vivax* infections are present in Sarangani Province, South Cotabato Province and Tawi-Tawi Province. These infections constitute continuous infectious reservoirs, could perpetuate transmission and threaten control and elimination efforts unless effectively treated. The threat of subpatent malaria should be considered when planning elimination in these provinces.
b. Microscopy in Tawi-Tawi Province and *P. falciparum*-specific HRP2 and *P. vivax*-specific LDH-based RDT in Sarangani Province and South Cotabato Province were not sensitive in identifying subpatent *P. falciparum* and *P. vivax* infections. A more sensitive diagnostic method with sensitivity and specificity comparable to PCR should be considered for use in these provinces.

c. The threat of importation of *P. malariae* and other *Plasmodium* species should be taken into account when planning malaria elimination in these provinces.

3.6. Limitations of the study

Low density of parasites in infected samples and the low number of infected humans among participants surveyed held back the results of this study. In addition, the questionnaire was not validated after it was translated to local dialects. Analyses of available participant data were limited to demographic and socio-cultural information. Factors associated with malaria were not determined in this study since the malaria positive humans were expected to be low and this small number might not be sufficient to detect evidence of association.

Chapter 4. Polymorphisms in the multi-drug resistance 1 (*pfmdr1*) and chloroquine resistance transporter (*pfcrt*) genes of *P. falciparum* isolates from Mindanao, The Philippines

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4.1. Background

The artemisinin combination therapy (ACT) policy was adopted by the Philippines in 2009 as cornerstone treatment for *P. falciparum* malaria in 53 endemic provinces (Department of Health Philippines, 2011, Chen et al., 2003). Artemisinin and its derivatives can effectively kill the asexual stages of *Plasmodium* and to some extent reduce gametocyte carriage while lumefantrine removes the remaining parasites in the circulation (Dondorp et al., 2010, Price et al., 1996, Ezzet et al., 2000). Combining artemisinin with a longer-acting partner drug, which has a different basis of genetic resistance, effectively protects both drugs from development of parasite resistance (Dye and Williams, 1997, White, 1999b). The emergence of parasites with reduced susceptibility to artemisinin compounds in Southeast Asia caused global concern because resistant parasites from Southeast Asia tend to spread rapidly on a geographic scale (Noedl et al., 2008, Noedl et al., 2009, Dondorp et al., 2009, Payne, 1987).

Prolonging the usefulness of artemether-lumefantrine (AL) is essential to achieving malaria elimination in the Philippines by 2020 (Department of Health Philippines, 2011). Chloroquine and amodiaquine have been used in the Philippines for several years before artemether-lumefantrine was introduced to the country. Years of chloroquine and amodiaquine use might have selected *P. falciparum* toward a genetic background that might affect the sensitivity of parasites to AL in the country. Chloroquine and amodiaquine were first used in the Philippines in the 1950s to treat malaria especially in areas that were hardly reached by vector control (Ejercito et al., 1954). Resistance to amodiaquine emerged in the Philippines in 1968 followed by resistance to chloroquine in 1971, which was believed to be a cross-reaction to amodiaquine pressure (Shute et al., 1972, Cabrera and Arambulo, 1977). Amodiaquine was used in the Philippines until the 1980s during which its *in vivo* and *in vitro* efficacy was reduced further by *P. falciparum* resistance (Smrkovski et al., 1985, Watt et al., 1987a, Long et al., 1987). Meanwhile, chloroquine remained the treatment of choice despite reports of reduced efficacy because this phenomenon was regarded as low-level chloroquine resistance (Watt et al., 1988, Baird et al., 1996a, Bustos et al., 1999,

Hatabu et al., 2003). Sulfadoxine and pyrimethamine (SP) as fixed combination was the second-line antimalarial treatment in the Philippines in the 1980s but resistance to this regimen developed by the late 1980s (Watt et al., 1987c). In 2002 the treatment of choice for *P. falciparum* malaria in the Philippines was changed to chloroquine, sulfadoxine and pyrimethamine combination. Artemether-lumefantrine was the second treatment option (World Health Organization, 2005). The chloroquine, sulfadoxine and pyrimethamine combination still showed 85% adequate clinical and parasitological response (ACPR) in field studies. This combination was retained as first line treatment only up to 2009 after which it was replaced with artemether-lumefantrine (World Health Organization, 2005, World Health Organization, 2010g). The Philippines still use chloroquine for treatment of non-falciparum malaria (Department of Health Philippines, 2010).

Chloroquine resistance is multigenic (Chen et al., 2002). This trait depends mainly on mutations in the *P. falciparum* chloroquine resistance transporter (*pfcrt*) gene and additional mutations in other genes such as the *P. falciparum* multidrug resistance (*pfmdr1*) gene (Fidock et al., 2000, Babiker et al., 2001, Wellems and Plowe, 2001). Before the *pfcrt* was identified in laboratory and field studies as the principal determinant of chloroquine resistance, the locus for this trait has been traced to chromosome 7 (Wellems et al., 1991, Djimde et al., 2001, Sidhu et al., 2002). The *pfcrt* encodes a 48.6-kDa protein, the PfCRT or chloroquine resistance transporter, which is composed of 424 amino acids. The PfCRT is located at the parasite's digestive vacuole and it has 10 predicted transmembrane domains (Fidock et al., 2000). Inside the digestive vacuole, it has been suggested that chloroquine exerts its antimalarial action by preventing the detoxification of haem following haemoglobin degradation (Goldberg et al., 1990). The build-up of haem is thought to be lethal to the parasite because it will increase the permeability of the sodium-potassium transporters and cause peroxidative damage (Ginsburg et al., 1998, Loria et al., 1999). The change in amino acid from lysine to threonine at position 76 of the *pfcrt* was found to be the key determinant in chloroquine resistance (Fidock et al., 2000). In the presence of *pfcrt* 76T mutation, the variant form of PfCRT on the digestive vacuole would allow chloroquine efflux, thereby reducing the effective therapeutic drug concentration

inside (Sanchez et al., 2005, Martin et al., 2009). It was postulated that the mutated PfCRT behaved as an ion channel allowing efflux of positively charged chloroquine following an electrochemical gradient (Warhurst, 2003). Alternatively, it was suggested that the PfCRT used an energy-dependent mechanism to remove chloroquine from the digestive vacuole (Krogstad et al., 1992, Lehane and Kirk, 2008).

It has been suggested that the development of the chloroquine resistant phenotype involved accumulation of nine mutations in the *pfcrt* gene, of which the lysine to threonine substitution at position 76 and the alanine to serine substitution at position 220 were considered most important in the development of resistance (Chen et al., 2001, Hastings et al., 2002, Warhurst, 2003). Other mutations in the *pfcrt* gene were regarded as compensatory for impaired PfCRT but the exact roles of these mutations remained to be elucidated (Johnson et al., 2004). While the *pfcrt* K76T mutation was found in resistant *P. falciparum* (Fidock et al., 2000), different *pfcrt* haplotypes associated with chloroquine resistance have been identified from several geographic regions. Chloroquine resistant *P. falciparum* isolates from Southeast Asia and Africa have *pfcrt* mutant haplotype CVIET in *pfcrt* codons 72 to 76 while those from South America and Papua New Guinea have *pfcrt* mutant haplotype VMNT, although the latter was also found in Asia and India (Fidock et al., 2000, Mehlotra et al., 2001). In the Philippines, two unique *pfcrt* mutations resulting from alanine to threonine substitution at position 144 (A144T) and leucine to tyrosine substitution at position 160 (L160Y) have been reported in chloroquine-resistant isolates collected from the Luzon Islands (Chen et al., 2003). These novel alleles were considered compensatory in the absence of the essential A220S mutation in chloroquine resistant isolates. This led to the suggestion of an independent focus of chloroquine resistance in the Philippines (Chen et al., 2005, Iwagami et al., 2009) in addition to the four known origins of chloroquine resistance in Southeast Asia, South America, and Papua New Guinea (Payne, 1987, Mehlotra et al., 2001). On the other hand, it has been suggested that the clinical efficacy of amodiaquine is influenced by novel mutations at *pfcrt* residues 72 to 76 especially the 76T allele (Warhurst, 2003, Ochong et al., 2003). In subsequent studies the *pfcrt* SVMNT haplotype was found to be associated with amodiaquine resistance (Dittrich et al., 2005, Beshir et al., 2010).

In addition to the *pfcrt* gene, single nucleotide polymorphisms (SNP) in the *pfmdr1* gene are also implicated in chloroquine resistance. The *pfmdr1* gene is located in chromosome 5 and encodes a 162-kDa protein, the PfMDR1 or P-glycoprotein homologue 1, which is located at the digestive vacuole of the parasite (Foote et al., 1989, Cowman et al., 1991). The protein PfMDR1 has two homologous domains, each with six transmembrane helices and a nucleotide-binding fold that is typical of the family of ATP-binding cassette proteins (Duraisingh and Cowman, 2005). Unlike the *pfcrt* gene, mutations in the *pfmdr1* gene and its amplification (Foote et al., 1989, Foote et al., 1990) are not sufficient to confer chloroquine resistance. Instead, it has been suggested that mutations in the *pfmdr1* gene modify levels of existing chloroquine resistance (Reed et al., 2000, Babiker et al., 2001). Chloroquine and amodiaquine positively selects for *P. falciparum* with *pfmdr1* 86Y mutant allele and the *pfmdr1* 86Y-184Y-1246Y haplotype respectively (Duraisingh et al., 1997, Humphreys et al., 2007). On the other hand, artemether-lumefantrine favorably selects for *P. falciparum* with *pfmdr1* 86N-184F-1246D haplotype resulting to susceptibility and increased survival of *P. falciparum* following treatment (Dokomajilar et al., 2006, Humphreys et al., 2007, Henriques et al., 2014). This would be of particular concern to countries that adopted ACT as first-line treatment against *P. falciparum* malaria.

With the implementation of the ACT drug policy in the Philippines in 2009, it would be important to determine the epidemiology and genetic background of *P. falciparum* in malaria endemic provinces that could affect ACT's usefulness locally. Hence, this chapter aimed to describe single nucleotide polymorphisms and haplotypes in the *pfcrt* and *pfmdr1* genes of *P. falciparum* isolates from Sarangani Province, South Cotabato Province, and Tawi-Tawi Province where these have not been previously studied.

4.2. Methods

Ethics statement

The LSHTM Ethics Committee (Reference No. 5712) and the Philippines National Ethics Committee have approved the participation of humans in this study in 2010. Prior written informed consent as described in Chapter 3 were secured from participants in the cross-sectional surveys and from respondents in municipal health centres.

Sample collection

Sixty-five *P. falciparum* isolates used in this study were diagnosed by PCR (Snounou et al., 1993, Humphreys et al., 2007) from 2,628 participants of cross-sectional surveys in Sarangani Province, South Cotabato Province and Tawi-Tawi Province in Mindanao, The Philippines from 2010 to 2013 as described in Chapter 3. The *P. falciparum* prevalence was expected to be low in these provinces; hence, municipal health centres were considered for collection of *P. falciparum* isolates for genotyping. As a pilot survey in municipal health centres of three and seven municipalities in Sarangani Province and South Cotabato Province respectively, blood spots on filter paper and information were collected from people presenting with symptoms of malaria from June to August 2010. From the municipal health centres, only 12 samples were collected of which 11 came from two municipalities in Sarangani Province and one from the municipality of T'boli in South Cotabato Province. Upon screening with nested PCR, two of the 12 samples from these municipal health centres were diagnosed with *P. falciparum* (Figure 4.4).

DNA extraction

The 67 samples used in this chapter have been previously screened and diagnosed with *P. falciparum* infection as described in Chapter 3. DNA extraction was repeated by punching 6mm diameter dried blood spot from each of the 67 samples and the

genomic DNA was extracted using QIAGEN® DNA mini kit (QIAGEN, Germany) following manufacturer's instruction. The *P. falciparum* 3D7 laboratory isolate was used as positive control and two blanks were used as negative control during DNA extraction. About 100 μL DNA was eluted and stored at -20C prior to molecular analyses.

Polymorphisms in *pfmdr1* gene

The *P. falciparum* isolates were genotyped in the *pfmdr1* for polymorphisms in codons 86, 184, 1034, 1042 and 1246 using primers and thermal cycling conditions as previously described (Humphreys et al., 2007, Dlamini et al., 2010). The *pfmdr1* haplotype NYSND found in *P. falciparum* 3D7 strain (Beshir et al., 2010) was used as wild type and positive control in this study. The PCR reaction mix for amplifying *pfmdr1* fragment 1 (codons 86 and 184) was the same for the hemi-nested PCRs amplifying *pfmdr1* fragment 3 (codons 1034 and 1042) and fragment 4 (codon 1246) except for the primers (Table 4.1). Five microliter of QIAGEN kit-extracted DNA was added to primary PCR mix, which has 1X NH4 buffer (Bioline), 2mM $MgCl_2$ (Bioline), 50μM each deoxyribonucleotide (dNTP) (Bioline), 200nM forward primer, 200nM reverse primer, and 1 unit (U) BIOTAQ™ DNA polymerase (Bioline). The final volume was adjusted to 25 μL with nuclease-free water. One microliter of the primary PCR product was used as template for the nested or hemi-nested PCR. Laboratory-grown 3D7 strain diluted in 1:100 was used as positive control. Negative controls in DNA extraction and no template PCR control were used as negative controls per assay. The reactions were carried out in G-storm GS2 thermal cycler (Labtech International Ltd.).

Table 4.1 Primers, cycling parameters and expected amplicon size for genotyping the *pfmdr1* gene.

Primer	Primer sequence	Cycling parameters	Product Size (bp)	References
For codons 86 and 184 (Primary PCR)		Initial denaturation at 94°C for 3 min followed by 30 cycles, each of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 65°C for 1 min; with final extension at 65°C for 5min	578	(Humphreys et al., 2007)
FN1/1 (forward)	AGGTTGAAAAAGAGTTGAAC			
REV/C1 (reverse)	ATGACACCACAAACATAAAT			
For codons 86 and 184 (Nested PCR)				
MDR2/1 (forward)	ACAAAAAGAGTACCGCTGAAT	Initial denaturation at 94°C for 3 min followed by 30 cycles, each of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 65°C for 1 min; with final extension at 65°C for 5min	534	
NEWREV1 (reverse)	AAACGCAAGTAATACATAAAGTC			
For codons 1034 and 1042 (Primary PCR)				
MDRFR3N1	GCATTTTATAATATGCATACTG	Initial denaturation at 94°C for 3 min followed by 30 cycles, each of denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec and extension at 65°C for 50 sec; with final extension at 65°C for 5min		(Dlamini et al., 2010)
MDRFR3R1	GGATTTCATAAAGTCATCAAC			
For codons 1034 and 1042 (Hemi-nested PCR)				
MDRFR3N2	GGTTTAGAAGATTATTTCTGTA	Same as in primary PCR	201	
MDRFR3R1	GGATTTCATAAAGTCATCAAC			

Table 4.1 Primers, cycling parameters and expected amplicon size for genotyping the *pfmdr1* gene (continuation).

Primer	Primer sequence	Cycling parameters	Product Size (bp)	References
For codon 1246 (Primary PCR)		Initial denaturation at 94°C for 3 min followed by 30 cycles, each of denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec and extension at 65°C for 50 sec; with final extension at 65°C for 5min		(Dlamini et al., 2010)
MDRFR4N1	CAAACCAATCTGGATCTGCAG		194	
MDRFR4R1	CAATGTTGCATCTTCTCTTCC			
For codon 1246 (Hemi-nested PCR)				
MDRFR4N2	GATCTGCAGAAGATTATACTG	Same as primary PCR	182	
MDRFR4R1	CAATGTTGCATCTTCTCTTCC			

Full-length sequence of the *pfcrt* gene

Hemi-nested primers were designed from the *P. falciparum* 3D7 laboratory isolate *pfcrt* gene (NCBI Accession No: 004328.2, Gene ID: 2655199) to amplify six fragments of the *pfcrt* gene as follows: *pfcrt* codons 72 to 97, codons 144 to 160, codon 220, codon 271, codons 326 to 334, and codons 356 to 371 (Table 4.2). The *pfcrt* gene was divided into smaller fragments for genotyping since parasite densities in the samples were expected to be low and intervening introns in the *pfcrt* gene would make amplification of larger fragments difficult. The PCR assays designed to amplify *pfcrt* codons 76-97, 144 to 160, 220, and 326 to 334 were optimised in the laboratory and PCR negatives were repeated. PCR assays designed to amplify *pfcrt* codons 271, 356 and 371 were not successful and thus, these codons were not included in the haplotype construction. The PCR mixes for primary and hemi-nested PCR assays were the same as described in *pfmdr1* amplification in Table 4.1 except for the primers. *Pfcrt* fragments were amplified as follows: denaturation at 95°C for 5 minutes followed by 30 cycles, each with 95°C denaturation for 30 sec, specific annealing temperature (Table 4.2) for 30 sec and 65°C extension for 1 min. These were followed by final extension at 65°C extension for 5 min. Where sample DNA was still available, PCR assays were repeated twice for each amplified fragment to confirm initial genotyping. Previously diagnosed *P. falciparum* positive samples that resulted to negative PCR results during genotyping were repeated for confirmation. Laboratory grown *P. falciparum* 3D7 strain was used as positive control and its *pfcrt* CVMNKHALLANTS haplotype was used as wild type for *pfcrt* codons 72-76, 97, 144, 148, 160, 220, 326, 333 and 334 in this study. The *P. falciparum* Dd2 (Foote et al., 1990) and the 7G8 (Burkot et al., 1984) resistant strains with CVIET and SVMNT haplotypes (Fidock et al., 2000) respectively were used as positive controls for mutant *pfcrt* haplotypes at codons 72-76.

Table 4.2. Primers designed for this study, annealing temperature and product size for determining polymorphisms in selected codons of the *pfcrt* gene.

Primer	Primer Sequence (5'-3')	Annealing Temperature (°C)	Product size (bp)
For codons 72 – 97			
CRT1N1	GGCTCACGTTTAGGTGGAGG	55	325
CRT1R1	GGTAGGTGGAATAGATTCTC		
CRT1N2	GTGGAGGTTCTTGTCTTGGTA	55	312
CRT1R1	GGTAGGTGGAATAGATTCTC		
For codons 144 - 160			
CRT2N1	GACCTTTTTAGGAACGACACC	56	167
CRT2R1	AAAGCAGAAGAACATATTAATAGG		
CRT2N2	AGGAACGACACCGAAGCTTTA	56	158
CRT2R1	AAAGCAGAAGAACATATTAATAGG		
For codon 220			
CRT3N1	CACTTATACAATTATCTCGGAGC	55	318
CRT3R1	AACTATTTCCCTTGTCATGTTTG		
CRT3N2	TCTCGGAGCAGTTATTATTGTTG	55	304
CRT3R1	AACTATTTCCCTTGTCATGTTTG		
For codons 326 - 334			
CRT4N1	GTCTTGGTATGGCTAAGTTATGTG	56	285
CRT4R1	TATTTCCTCTTGTATGTATCAACG		
CRT4N2	GATTGTGACGGAGCATGGGTAA	56	259
CRT4R1	TATTTCCTCTTGTATGTATCAACG		

Viewing and sequencing of amplicons

The amplicons were viewed with 2% agarose in 0.5X TBE buffer, sequenced using Big Dye® Terminator v3.1 cycle sequencing kit (Applied Biosystems), and analyzed in ABI 3730 sequencer (Applied Biosystems). The chromatograms were aligned and viewed using Geneious® software version 7.0 (Biomatters, New Zealand) and Chromas software version 1.61 (Technelysium Pty Ltd., Queensland).

Haplotype analyses

Haplotypes were separately constructed for *pfmdr1* codons 86, 184, 1034, 1042 and 1246 and *pfcrt* codons 72 to 76, 144, 148, 160, 220, 326, 333 and 334 in *P. falciparum* isolates with complete data. *P. falciparum* isolates with mixed haplotypes at *pfcrt* codons 72-76 were excluded.

4.3. Results

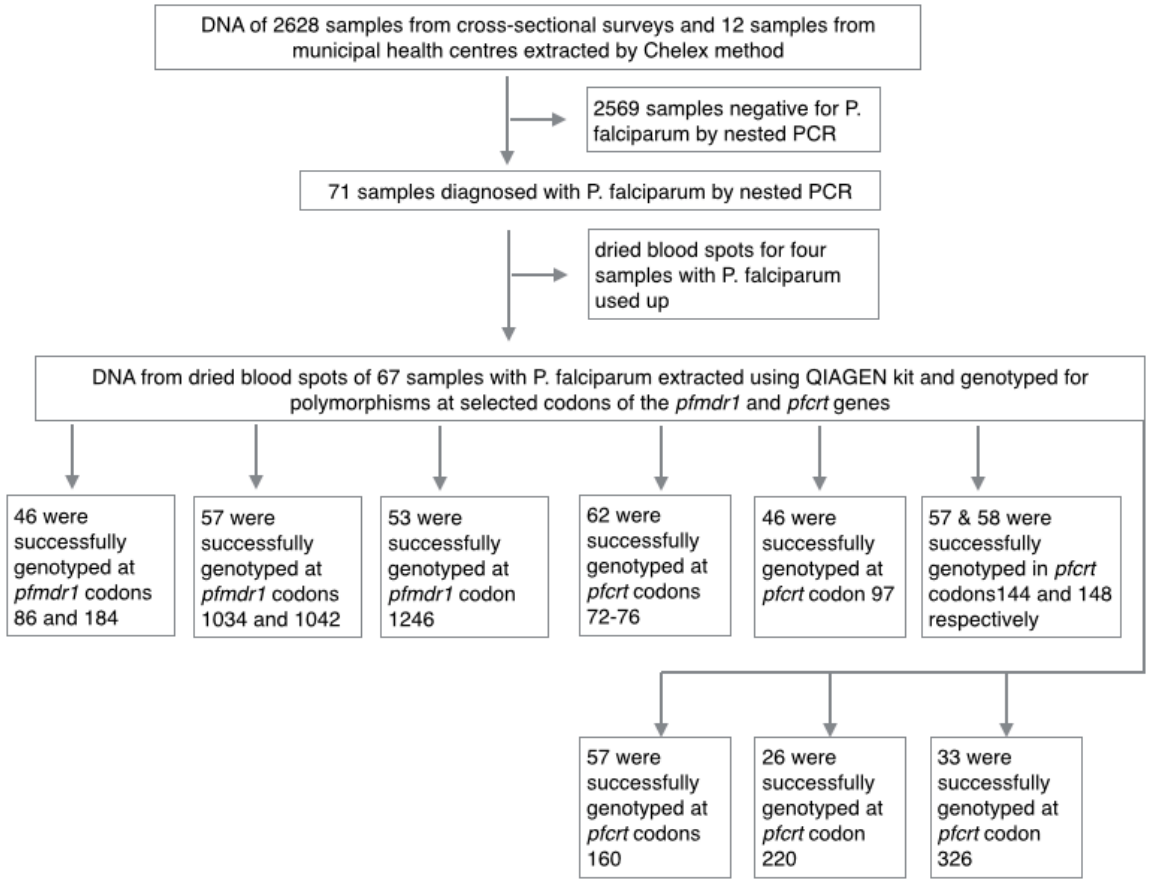


Figure 4.1. Summary for selection of *P. falciparum* isolates from Mindanao, The Philippines for *pfmdr1* and *pfcrt* molecular genotyping.

Sixty-seven (67) *P. falciparum* isolates from Mindanao were genotyped for single nucleotide polymorphisms in selected codons of the *pfmdr1* and *pfcrt* genes. Sixty-five of these isolates were from people who attended the cross-sectional surveys in three endemic provinces in Mindanao while two of the *P. falciparum* isolates were from individuals seeking diagnosis and treatment at municipal health centres in the Provinces of Sarangani and South Cotabato (Figure 4.1). It was expected that parasite density would be low in these samples and thus, PCR primers were designed to amplify shorter fragments of the *pfcrt* gene containing polymorphic codons.

Characterisation of *pfmdr1* alleles and haplotypes

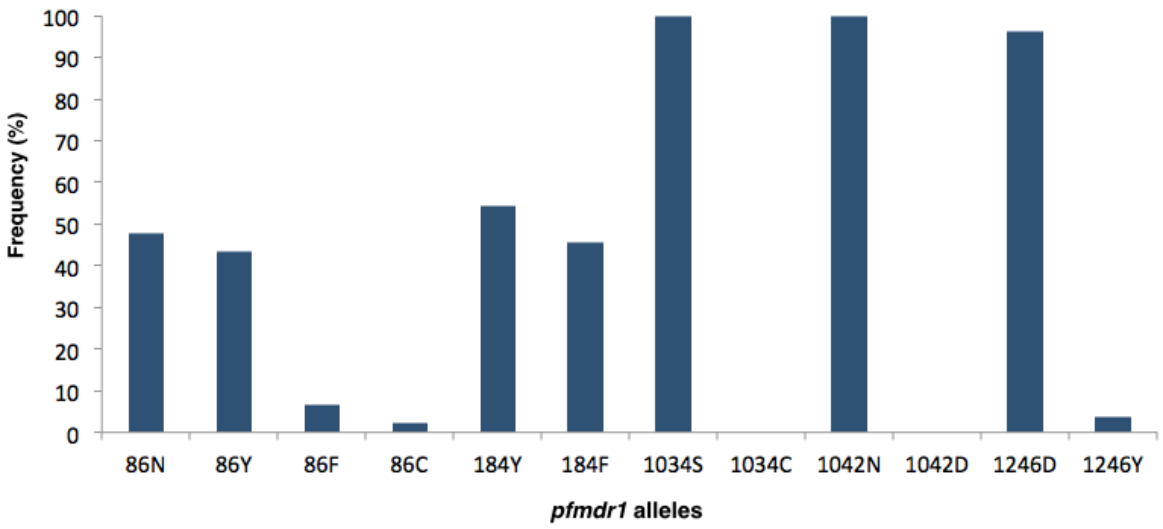


Figure 4.2. Frequency of *pfmdr1* alleles at positions 86, 184, 1034, 1042 and 1246 in selected *P. falciparum* isolates from Mindanao, The Philippines.

The *pfmdr1* codons 86 and 184 were successfully genotyped in 46 *P. falciparum* isolates from Mindanao. The *pfmdr1* wild type 86N and mutant 86Y alleles were found in 47.8% (22) and 43.5% (20) of *P. falciparum* isolates respectively (Figure 4.2). Two uncommon *pfmdr1* mutant 86F and 86C alleles were identified in 6.5% (3) and 2.2% (2) *P. falciparum* isolates from South Cotabato respectively (Figures 4.2). The *pfmdr1* 86F and 86C alleles have two base mutations **TT**T and **TG**T (mutated base in bold font) respectively compared to the *pfmdr1* wild-type 86N allele coded by AAT (Figure 4.4). These samples were repeatedly genotyped in the same codon where variant alleles were observed for validation but there was poor reproducibility in repeat PCR assays due to low parasite density. The *pfmdr1* 184Y and 184F alleles were found in 54.5% (25) and 45.6% (21) of the *P. falciparum* isolates successfully genotyped. Only *pfmdr1* wild-type alleles 1034S and 1042D were observed in 57 *P. falciparum* isolates successfully genotyped in these codons. The frequency of *pfmdr1* wild-type 1246D

allele was higher than that of the mutant allele 1246Y at 96.2% (51) and 3.8% (2) respectively (Figure 4.2).

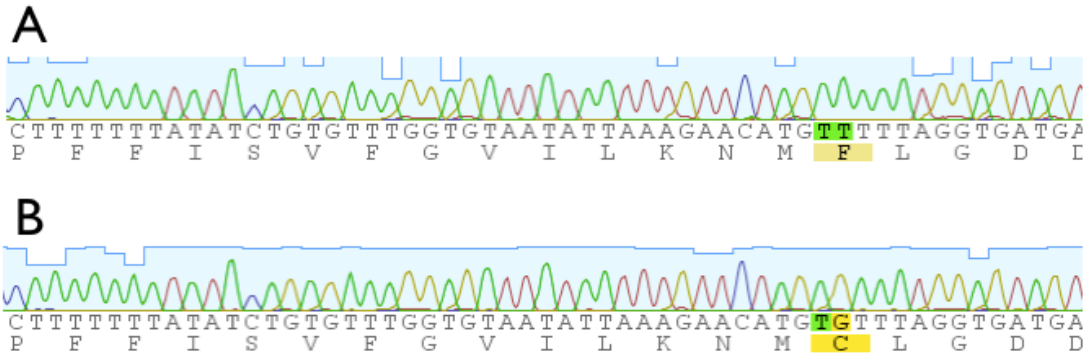


Figure 4.3. Chromatograms of mutant *pfmdr1* 86F (A) and 86C (B) alleles identified in *P. falciparum* isolates from Mindanao, The Philippines. The mutated amino acid is highlighted in yellow. The codon for the *pfmdr1* wild type 86N is AAT.

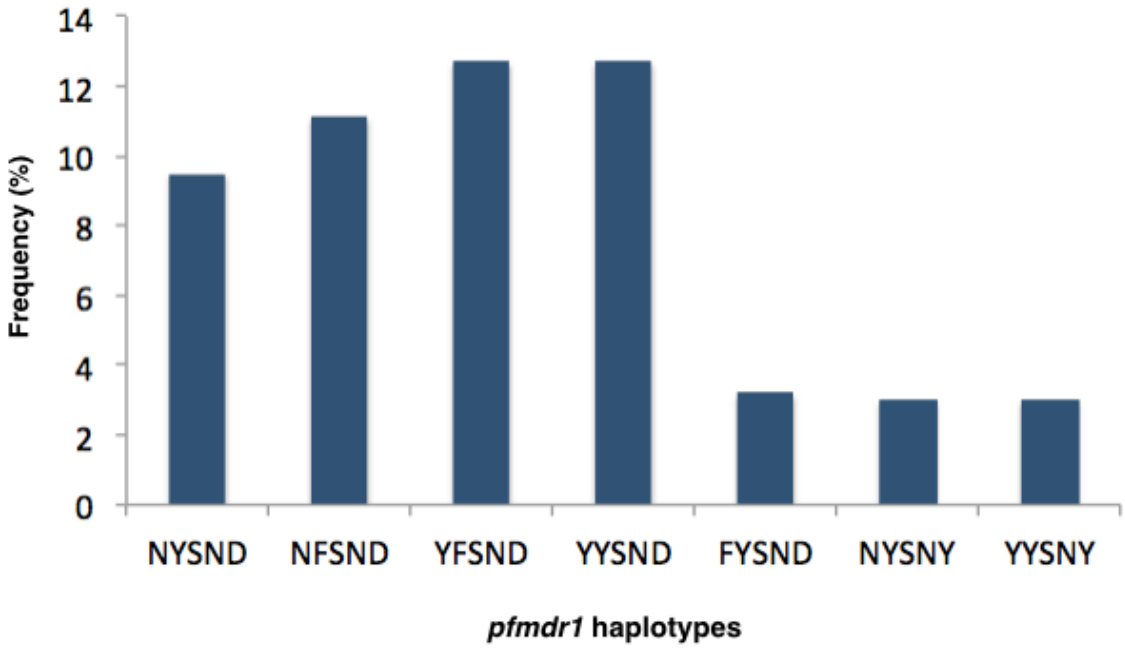


Figure 4.4. Frequency of haplotypes constructed from *pfmdr1* codons 86, 184, 1034, 1042 and 1246 of 33 *P. falciparum* isolates from Mindanao, The Philippines.

Seven haplotypes were constructed from *pfmdr1* codons 86, 184, 1034, 1042 and 1246 of 33 *P. falciparum* isolates with complete data (Figure 4.4). *Pfmdr1* codons 1034 and 1042 were invariant in these *P. falciparum* isolates but these were included in the haplotype analysis. The *pfmdr1* wild-type 86N-184Y-1034S-1042N-1246D or NYSND and mutant N**F**SND (mutated amino acid in bold font) haplotypes were found in 9.5% (6) and 11.1% (7) of *P. falciparum* isolates with complete data respectively. The *pfmdr1* mutant **Y**YSND and **YF**SND haplotypes were each found in 12.7% (8) of *P. falciparum* isolates successfully genotyped. The *pfmdr1* single mutant **F**YNSD and NYSN**Y** and double mutant **Y**YSN**Y** haplotypes were observed in 3.2% (2), 3.0% (1) and 3.0% (1) respectively of the *P. falciparum* isolates successfully genotyped. The *pfmdr1* double mutant **Y**YSN**Y** was identified from a patient with fever and seeking diagnosis and treatment at the Municipal Health Centre of Maasim, Sarangani Province.

Characterisation of *pfcrt* alleles and haplotypes

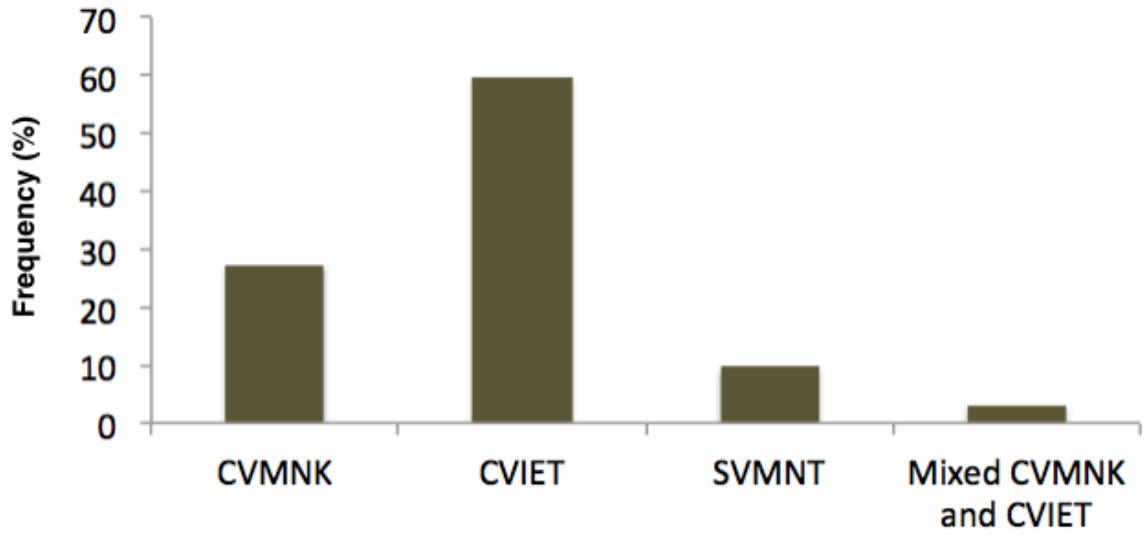


Figure 4.5. Frequency of haplotypes constructed for *pfcrt* codons 72 to 76 among 62 *P. falciparum* isolates from Mindanao, The Philippines.

The *pfcrt* haplotype was successfully constructed for codons 72 to 76 in 62 *P. falciparum* isolates with complete data (Figure 4.5). The *pfcrt* wild-type CVMNK was identified in 27.4% (17) of *P. falciparum* isolates while the *pfcrt* mutant CV**IET** and **S**$_{[AGT]}$VMN**T** (mutated amino acid in bold font) were seen in 59.7% (37) and 9.7% (6) of *P. falciparum* isolates respectively. Two of the 62 *P. falciparum* isolates have mixed *pfcrt* CVMNK and CVIET haplotypes. The *pfcrt* wild-type CVMNK and mutant CVIET haplotypes were seen in the three provinces surveyed while *pfcrt* mutant **S**$_{[AGT]}$VMN**T** was absent among *P. falciparum* isolates from South Cotabato Province. This was the first confirmation of *pfcrt* wild-type CVMNK and mutant CVIET haplotypes in Mindanao, The Philippines (Figure 4.6).

Aside from *pfcrt* codons 72, 74-76, polymorphisms in *pfcrt* codons 97, 144, 148, 160, 220, 326, 333 and 334 were also described in this study in an attempt to construct the full-length sequence of the *pfcrt* gene among *P. falciparum* isolates from Mindanao. We were not successful in amplifying polymorphic codons 271, 356 and 371 and thus, these were excluded from the *pfcrt* haplotype construction. The two *P. falciparum* isolates from Sarangani Province and Tawi-Tawi Province that showed real mix *pfcrt* genotype (Figure 4.5) were excluded in the construction of *pfcrt* haplotypes for codons 72-76, 97, 144, 148, 160, 220, 326, 333 and 334. The *pfcrt* codons 97, 144, 148 and 160 were invariant among *P. falciparum* isolates successfully genotyped. Meanwhile, 69.2% (18) and 30.8% (8) of *P. falciparum* isolates have *pfcrt* wild-type 220A and mutant 220S alleles respectively. About 66.7% (22) of *P. falciparum* isolates have the *pfcrt* wild-type 326N allele while 24.8% (8) and 9.1% (3) have *pfcrt* mutant 326D and 326S alleles respectively. These *pfcrt* alleles were confirmed in two or three repeated PCR and sequencing assays. Seven *pfcrt* haplotypes were constructed for polymorphic codons 72, 74-76, 97, 144, 148, 160, 220, 326, 333 and 334 in seven *P. falciparum* isolates from Mindanao with complete data (Table 4.3). These haplotypes have been confirmed in repeated PCR and sequencing. None of the *pfcrt* haplotypes in *P. falciparum* isolates from Mindanao were similar to *pfcrt* haplotypes of *P. falciparum* 3D7 (control) and the *P. falciparum* strains P2a, P2b and E1 that were previously reported in the Philippines.

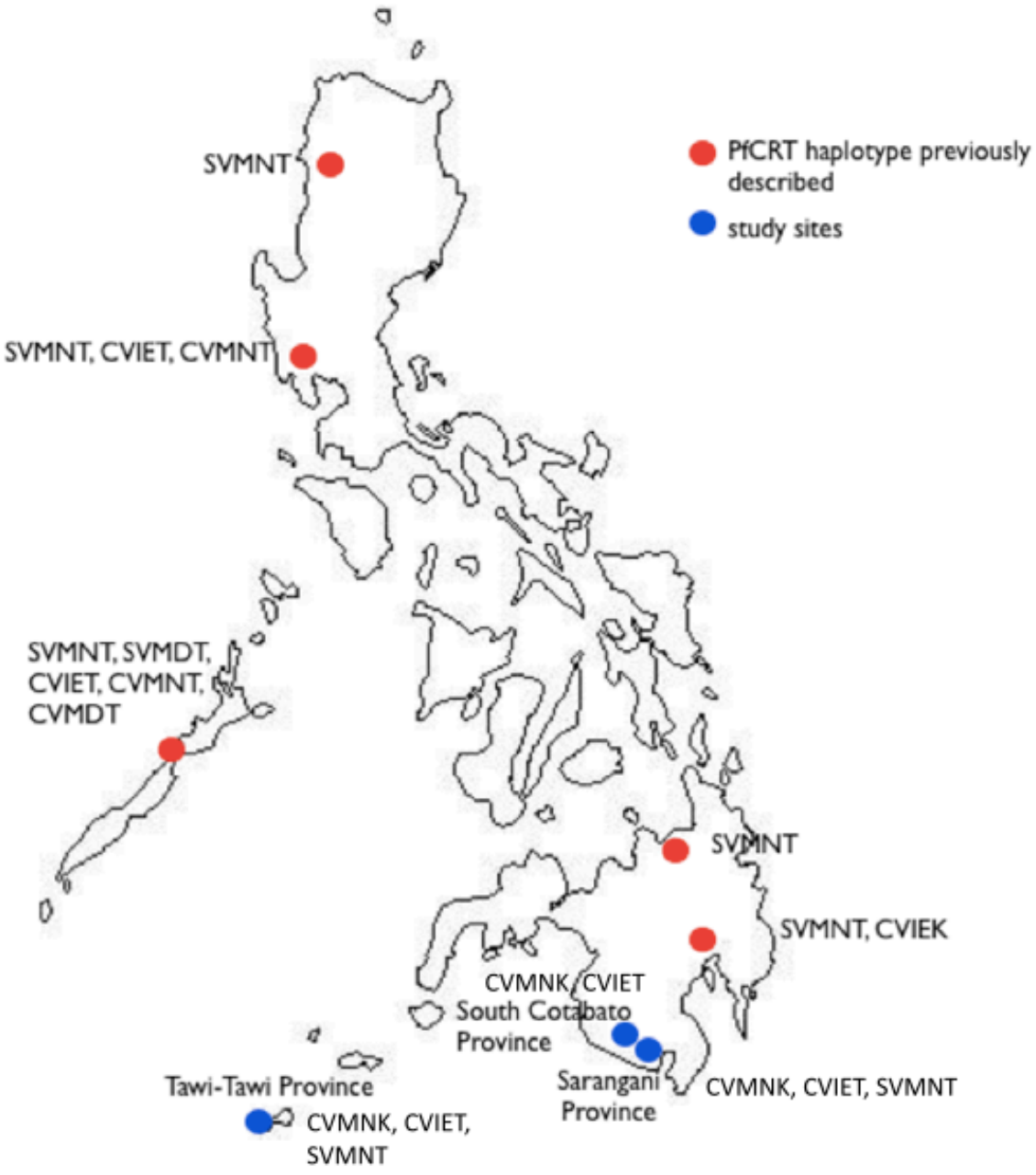


Figure 4.6. Distribution of *pfcrt* codons 72 to 76 haplotypes in the Philippines showing previously described haplotypes in red dots (Chen et al., 2003, Chen et al., 2005) and the identified haplotypes in this study in blue dots.

Table 4.3. Polymorphisms in the PfMDR1 and PfCRT of isolates from Mindanao, The Philippines.

	PfMDR1					PfCRT										
Isolates	**86**	**184**	**1034**	**1042**	**1246**	**72**	**74**	**75**	**76**	**97**	**144**	**148**	**160**	**220**	**326**	**333**	**334**
3D7	N	Y	S	N	D	C	M	N	K	H	A	L	L	A	N	T	S
P2a*	Y	Y	S	N	D	C	N	N	T	H	T	-	Y	A	D		
P2b*	Y	Y	S	N	D	S	M	N	T	H	T	-	Y	A	D		
E1a**	N	F	C	D	D	C	I	E	T	H	A	-	L	S	S		
TGP 075	N	Y	S	N	Y	C	M	N	K	H	A	L	L	A	N	T	S
TT 489	N	F	-	-	D	C	M	N	K	H	A	L	L	A	S	T	S
TGP 084	Y	Y	S	N	D	C	I	E	T	H	A	L	L	A	N	T	S
TMJ 068	Y	F	S	N	-	C	I	E	T	H	A	L	L	A	S	T	S
TT 290	N	Y	S	N	D	C	I	E	T	H	A	L	L	S	S	T	S
TMJ 080	Y	Y	S	N	D	S	M	N	T	H	A	L	L	S	D	T	S
TMK 050	N	F	S	N	D	S	M	N	T	H	A	L	L	A	N	T	S

*Isolates with novel *pfcrt* alleles: Thr-144 and Tyr-160; **Southeast Asian allelic type described in the Philippines (Chen et al., 2003)
Amino acids different from the wild type (3D7) are shaded in grey.
Isolates TGP075, TMJ 068, TMJ 080 and TMK 050 were from Sarangani Province.
Isolates TT 489 and TT 290 were from Tawi-Tawi Province.

4.4 Discussion

The Philippines has launched the national malaria elimination campaign and adopted the ACT drug policy to further reduce malaria in its 53 endemic provinces. Ye, little was known about the genetic background of *P. falciparum* that might affect the usefulness of artemether-lumefantrine combination therapy (ACT) in the country. Studies have shown that polymorphisms in the *pfcrt* and *pfmdr1* genes influence susceptibility of *P. falciparum* to some artemisinin derivatives and their partner drugs. Incidentally, these genes were involved in chloroquine and amodiaquine resistance (Fidock et al., 2000, Reed et al., 2000, Babiker et al., 2001). These 4-aminoquinoline compounds have been used for treatment of *P. falciparum* infection since the 1950s and might have exerted selection pressure on the parasite. This chapter aimed to describe polymorphisms in the *pfcrt and pfmdr1* genes of selected *P. falciparum* isolates from three malaria endemic provinces in Mindanao, The Philippines.

In this study new PCR strategies were used to describe the full sequence of the *pfcrt* gene in *P. falciparum* isolates collected from subpatent infections in Mindanao. This attempt was partially successful as results were held back by the sample size, quality of the DNA, the volume of the blood used in DNA extraction, the introns of the *pfcrt* gene and the parasite density in infected samples. The mutant CV**IET** (mutated amino acid in bold font) was the major *pfcrt* haplotype at codons 72-76 among *P. falciparum* isolates from the three provinces surveyed. This might be suggestive of continuing chloroquine pressure since this drug is still used to treat non-falciparum malaria in the Philippines despite its withdrawal for treatment of *P. falciparum* infection in 2009 (Department of Health Philippines, 2010). It was possible that *P. falciparum* might be subjected to suboptimal chloroquine concentration (Bloland, 2001) if it was mistaken as *P. vivax* or if co-infecting *P. falciparum* was not diagnosed because of subpatent infection. This study was the first to confirm presence of *pfcrt* wild-type CVMNK and mutant CV**IET** haplotypes in Mindanao. The mutant **S**VMN**T** haplotype, which has the lowest frequency compared to CVMNK and CV**IET** haplotypes in this study has been previously reported in Mindanao and considered to be the dominant *pfcrt* 72-76 haplotype in the

Philippines (Chen et al., 2003, Chen et al., 2005, Hatabu et al., 2009, Takahashi et al., 2012). The history of chloroquine pressure might be different in Mindanao compared to other regions in the Philippines. In this study none of the *P. falciparum* isolates with *pfcrt* mutant 76T allele has the mutant 144T and 160Y alleles previously described in Philippine *P. falciparum* isolates with mutant *pfcrt* K76T allele but without the mutant 220S allele that went with it (Chen et al., 2003). D. Warhurst (personal communication) suggested that there might be other compensatory mutations in the *pfcrt* gene in these isolates. Further studies and improved optimisation of PCR assays targeting other codons in the *pfcrt* gene would be needed to demonstrate this.

The *pfmdr1* gene showed allelic variations at codons 86, 184 and 1246. In this study the frequency of *pfmdr1* wild-type 86N allele (47.8%), which was associated with increased susceptibility to chloroquine but indicative of reduced susceptibility to mefloquine, artesunate, artemisinin and lumefantrine (Duraisingh et al., 2000, Pickard et al., 2003), was higher than the observed frequency of *pfmdr1* mutant 86Y allele (43.5%). The latter was associated with chloroquine resistance but increased susceptibility to mefloquine, halofantrine, lumefantrine and artemisinin derivatives (Duraisingh et al., 1997, von Seidlein et al., 1997, van Schalkwyk et al., 2013, Conrad et al., 2014). Two uncommon *pfmdr1* alleles 86F and 86C were observed in three and one *P. falciparum* isolate from South Cotabato Province, respectively. Dlamini et al. (2010) has reported the *pfmdr1* 86F allele in field isolates of *P. falciparum*. To this author's knowledge, the *pfmdr1* 86C allele has not been reported elsewhere. It has been suggested that withdrawal of chloroquine might exert fitness cost on the mutant *pfmdr1* 86Y allele, which would give likely give rise to mutant 86F (TTT) and 86C (TGT) alleles since each would only require single base mutation from the mutant 86Y (TAT) allele. The *pfmdr1* NFSND haplotype has been associated with prolonged parasite survival with artemether-lumefantrine treatment (Dokomajilar et al., 2006, Humphreys et al., 2007, Gadalla et al., 2011, Henriques et al., 2014). The presence of *pfmdr1* NFSND haplotype circulating as subpatent *P. falciparum* infections in Mindanao might have future implications to the use of ACT policy in this region.

4.5. Conclusions

These findings provided baseline data on SNPs and haplotypes in the *pfmdr1* and *pfcrt* genes circulating among subpatent *P. falciparum* infections in Mindanao. Further studies are needed to determine the implications of mutant *pfmdr1* NFSND haplotype and mutant *pfcrt* CVIET and SVMNT haplotypes in the ACT policy currently adopted by the Philippines and the continued use of chloroquine for treatment of non-falciparum malaria.

4.6. Limitations of the Study

Attempts to completely characterise the *pfcrt* gene were held back by quality of the DNA, the introns of the *pfcrt* gene and the low parasite density in the samples screened. Optimisation of primers designed to amplify codons 271, 356 and 371 and the PCR assays would have allowed genotyping of these codons.

Chapter 5. Single nucleotide polymorphisms in the multidrug resistance (*pvmdr1*) gene of *Plasmodium vivax* from Mindanao, The Philippines

5.1. Background

Plasmodium vivax is a human malaria parasite that originated from an ancient parasite lineage and switched hosts from non-human primates to hominids in Africa (Liu et al., 2014). It is most prevalent in South and Central America, Middle East, Asia and the Western Pacific but the public health burden of *P. vivax* has been overshadowed by high mortality and widespread drug resistance associated with *P. falciparum* (Mendis et al., 2001, Guerra et al., 2010). The impact of *P. vivax* on public health has been re-evaluated upon recognising that this species is more difficult to eliminate than *P. falciparum* because *P. vivax* can form dormant hypnozoites in the liver (Krotoski et al., 1982). Activation of hypnozoites leads to relapse infections at different intervals from the initial infection (Chen et al., 2007, Imwong et al., 2007). Infection with *P. vivax* may become severe (Barcus et al., 2007, Tjitra et al., 2008) although in most cases the disease is rarely life threatening. Unlike *P. falciparum* gametocytes of *P. vivax* have higher transmission potential at subpatent infection (Boyd and Kitchen, 1937). This is challenging in areas where malaria burden has been reduced by control measures and yet subpatent infections continue to persist undetected (Harris et al., 2010, Van den Eede et al., 2011). In some endemic areas the Duffy negative trait no longer protects the population against *P. vivax* infection because the parasite has developed alternative pathways to invade erythrocytes (Ryan et al., 2006, Mendes et al., 2011).

In the Philippines about 20% of malaria infections are caused by *P. vivax* (World Health Organization, 2014a). These *P. vivax* infections are treated with chloroquine and primaquine following recommendations of the WHO (Department of Health Philippines, 2010). Chloroquine is a 4-aminoquinoline compound that has been used to treat *P. vivax* infections since the 1940s (Coatney, 1963). In the absence of parasite resistance, chloroquine can inhibit polymerisation of toxic haem during haemoglobin degradation in the *Plasmodium* digestive vacuole resulting to parasite death (Sullivan et al., 1996). As previously mentioned *P. vivax* can relapse following activation of dormant hypnozoites in the liver (Krotoski et al., 1982, White, 2011). Radical cure from *P. vivax* malaria is only achieved following treatment with primaquine (Hill et al., 2006,

World Health Organization, 2010b). Primaquine is an 8-aminoquinoline compound that kills mature gametocytes of *P. falciparum* essential for transmission (Burgess and Bray, 1961) and latent liver stages of *P. vivax* responsible for relapse infection (Warhurst, 1984, Pukrittayakamee et al., 1994). The mechanism of action of primaquine is not well understood. It is suggested that metabolites of primaquine reacts with hemoglobin and oxygen resulting to formation of methemoglobin and reactive oxygen species through cytochrome P450 (Strother et al., 1981, Fletcher et al., 1988, Ganesan et al., 2009). In glucose-6-phosphate dehydrogenase (G6PD) deficient individuals, primaquine metabolites cause oxidative lysis of erythrocytes and may become fatal (Burgoine et al., 2010, Ganesan et al., 2012). This risk prompted the necessity for screening G6PD deficiency before any primaquine administration (World Health Organization, 2010b).

Chloroquine-resistant (CQR) *P. vivax* was reported 30 years after the emergence of chloroquine-resistant *P. falciparum* at the Thailand-Cambodia border in 1957 (Payne, 1987, Rieckmann et al., 1989). One possible reason for this late onset of *P. vivax* resistance to chloroquine was the early appearance of its gametocytes during the course of infection, which were more likely transmitted before chloroquine was given (McKenzie et al., 2002, Douglas et al., 2010). Another likely reason was the use of primaquine that kills hypnozoites of *P. vivax* thereby reducing exposure of *P. vivax* to chloroquine pressure in relapse infections. Following its first report, chloroquine-resistant *P. vivax* has become widespread in Southeast Asia and South America (Baird et al., 1991, Marlar et al., 1995, Garg et al., 1995, Baird et al., 1996g, Rijken et al., 2011). In 2009 chloroquine was no longer used to treat *P. vivax* infection in Vanuatu, Solomon Islands, Papua New Guinea and Papua, Indonesia due to development *P. vivax* resistance (Douglas et al., 2010).

Chloroquine resistance in *P. vivax* was defined as the recurrence of *P. vivax* within 28 days after complete duration of chloroquine treatment (Baird et al., 1997a). This resistance phenotype needs to be distinguished from relapse or activation of dormant hypnozoites and from recrudescence or recurrence of infection from subpatent parasitaemia (Chen et al., 2007). This difficulty in identifying true treatment failure

from relapse and recrudescent *P. vivax* infections was challenging in clinical trials such that molecular surveillance seemed a robust option in monitoring *P. vivax* responses to chloroquine. It has been suggested that development of CQR *P. vivax* was different from development of CQR in *P. falciparum* (Baird et al., 1997b). While the *pfcrt* gene played a central role in the development of chloroquine resistance in *P. falciparum* (Fidock et al., 2000), its orthologue the *pvcg10* gene did not mediate chloroquine resistance in *P. vivax* (Nomura et al., 2001, Suwanarusk et al., 2007). Instead it was the *P. vivax* multidrug resistance gene-1 (*pvmdr1*), which was implicated in the development of chloroquine resistance (Brega et al., 2005). The *pvmdr1* locus was found in chromosome 10. This gene is a single open reading frame and is 4,392 bp long. It encodes a P-glycoprotein composed of 1,464 amino acids, which belongs to a family of ATP-binding transporters. Gene amplification and single nucleotide polymorphisms (SNPs) in selected codons of the *pvmdr1* influenced susceptibility of *P. vivax* to several antimalarials (Brega et al., 2005, Suwanarusk et al., 2008). *Pvmdr1* wild type alleles 91N and 189Y and mutant alleles Y976F and F1076L were observed in *P. vivax* from areas of varying endemicity (Brega et al., 2005). Of the two mutant alleles, the tyrosine to phenylalanine substitution at *pvmdr1* position 976 was found to be associated with *in vivo* and *in vitro* chloroquine resistance of *P. vivax* isolates from Papua New Guinea, Indonesia, Thailand and Cambodia (Suwanarusk et al., 2008, Marfurt et al., 2008, Lin et al., 2013).

The Philippines is progressing towards malaria elimination but there are limited data on *P. vivax* responses to chloroquine (Baird et al., 1996a, World Health Organization, 2005). This exploratory chapter aimed to describe single-nucleotide polymorphisms in the *pvmdr1* gene of selected *P. vivax* isolates from Mindanao, The Philippines, which has not been previously described in the Philippines.

5.2. Methods

Sample collection and DNA extraction

Fifty-nine (59) samples from three provinces of Mindanao previously diagnosed with *P. vivax* were selected for *pvmdr1* genotyping. These 59 samples were positive for single infections with *P. vivax* using speciation and genotyping PCR as described in Chapter 3 and this chapter. DNA of each sample was re-extracted from blood spots on Whatman® 3MM filter paper using QIAamp® DNA mini kit (Qiagen, Germany) following manufacturer's instructions.

DNA controls

The Malaria Reference Laboratory (MRL) of the London School of Hygiene & Tropical Medicine provided the reference clinical isolates used as positive controls for *P. vivax mdr1* genotyping. These were as follows (MRL identification/country of origin): MRL 12/450 (French Guyana), MRL 12/525 (Peru), MRL 12/1103 (Indonesia), MRL 12/985 (India), MRL 12/321 (Somalia), MRL 12/ 594 (Solomon Islands) and MRL 12/750 (Pakistan).

Primers and PCR cycling conditions

The parasite density was expected to be low in *P. vivax* samples from Mindanao and thus, this exploratory chapter did not attempt to amplify the full *pvmdr1* gene. Instead hemi-nested primers were designed to amplify regions targeting *pvmdr1* codons 91, 976 and 1076 where polymorphisms have been previously reported. These codons corresponded to *pfmdr1* codons 86, 1034 and 1042 and were previously described in *P. vivax* isolates. The PCR primers were designed from the *pvmdr1* gene of the chloroquine-sensitive *P. vivax* strain Salvador-1 (NCBI Accession No. AY618622; Gene ID 5473000) (Contacos et al., 1972) (Table 5.1). The annealing temperature for each hemi-nested PCR assay was determined using temperature gradient PCR. The primers,

PCR annealing temperature and expected amplicon size were given in Table 5.1. The PCR reaction mixture was the same for all PCR assays amplifying *pvdmr1* codons 91, 976 and 1076. For the primary PCR, 5µL DNA were added to the PCR mix containing 1X NH4 buffer (Bioline), 1.5mM $MgCl_2$ (Bioline), 50µM each deoxyribonucleotide (dNTP) (Bioline), 200nM forward primer, 200nM reverse primer and 1 unit (U) BIOTAQ™ DNA polymerase (Bioline). The final volume for each reaction was adjusted to 25µL by adding sterile nuclease-free water. One microliter of the primary PCR product was used as template for the hemi-nested PCR assay using the same PCR reaction mixture as the primary PCR, except for the primers provided in Table 5.1. The *P. vivax* reference clinical isolates provided by the LSHTM Malaria Reference Laboratory were used as positive controls while the negative controls during sample DNA extraction and PCR no template controls (NTC) were used as negative controls in the PCR assay. Except for the annealing temperature, the cycling conditions were the same for all PCR assays as follows: initial denaturation at 95°C for 5 min followed by 30 cycles, each of denaturation at 95°C for 30 sec, specific annealing temperature for 30 sec and extension at 72°C for 1 min; and final extension at 72°C for 5min. Hemi-nested PCR products were run in 2% agarose using 0.5X Tris-Borate-EDTA (TBE) to view amplicons. The PCR assay was valid if the positive controls were amplified and there was no evidence of contamination in the negative controls. Samples previously diagnosed with *P. vivax* but were negative in PCR assays described here for *pvmdr1* codons 976 and 1076 were repeated.

Table 5.1. Target codons, primer sequences designed for this study, PCR annealing temperature and expected amplicon sizes for genotyping sequence polymorphisms in the *pvmdr1* gene.

Target codon	Primer	Primer Sequence	Annealing Temp (°C)	Amplicon Size (bp)
Codon 91	Primary PCR			
	P91N1	CCGTCAAGTCATAGGAAGCTGTT	62	195
	P91R1	GAAGCTCGAAATGAAGGACAGAAT		
	Hemi-nested PCR			
	P91N2	TAGGAAGCTGTTGGGGGTGT	62	184
	P91R1	GAAGCTCGAAATGAAGGACAGAAT		
Codon 976	Primary PCR			
	P976N1	GACCAGGATAGTCATGCCCCA	60	256
	P976R1	TGACTCGCTTCTTCTCTACATCC		
	Hemi-nested PCR			
	P976N2	ATGCCCCAGGATTGCTGTCAG	60	243
	P976R1	TGACTCGCTTCTTCTCTACATCC		
Codon 1076	Primary PCR			
	P1076N1	ACGGGCTGGAGGATTACTTCTG	62	241
	P1076R1	TTCCCGGCGTAGCTTCCCG		
	Hemi-nested PCR			
	P1076N2	GGAGGATTACTTCTGCACACTGAT	62	234
	P1076R1	TTCCCGGCGTAGCTTCCCG		

Sequencing and analyses of PCR amplicons

PCR amplicons were directly sequenced using the BigDye® Terminator V3.1 cycle sequencing kits (Applied Biosystems) and analysed on an ABI 3730 sequencer (Applied Biosystems). The sequence chromatograms were analysed using Geneious® version 6.1.6 (Biomatters, New Zealand) and Chromas version 1.61 (Technelysium). The nucleotide Basic Local Alignment Search Tool (BLAST) program of the National Center for Biotechnology Information was used to compare and confirm identity of sequences as *P. vivax*.

Haplotype analysis

Haplotypes were constructed for codons 91, 976 and 1076 in *P. vivax* isolates with complete data. These were compared to the haplotypes of the *P. vivax* reference clinical isolates from the MRL and the haplotype of the chloroquine-sensitive wild type *P. vivax* Salvador-1. Genotyping was repeated twice for validation.

5.3. Results

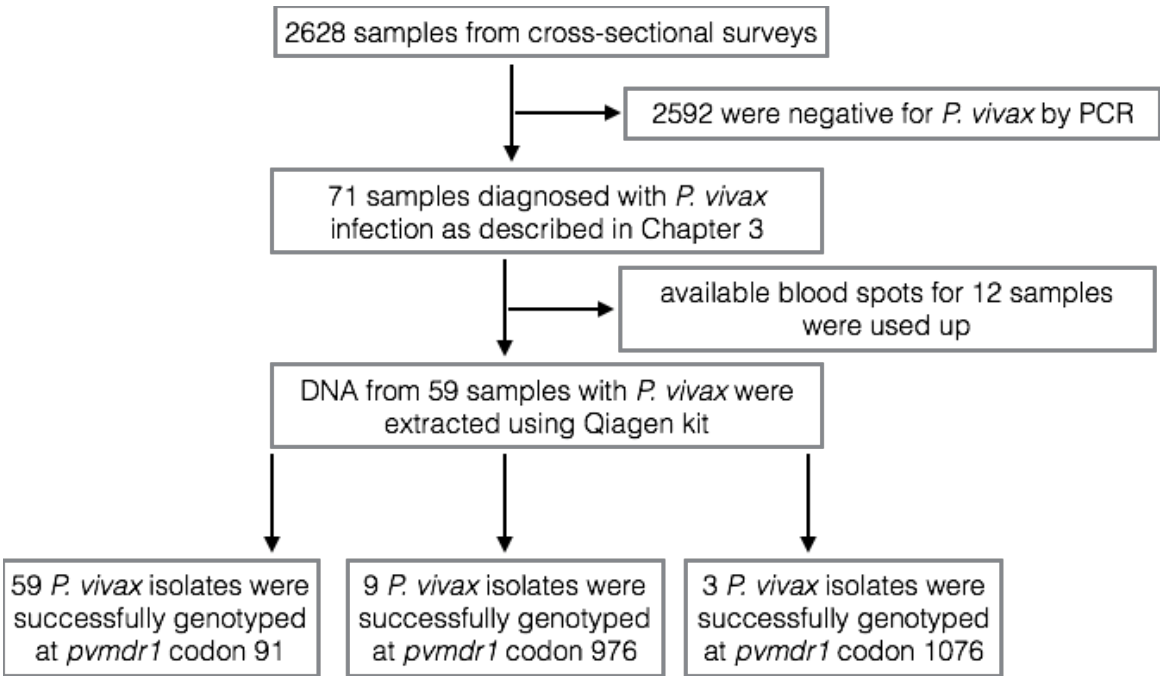


Figure 5.0. Summary of selection of *P. vivax* isolates from Mindanao, The Philippines for genotyping at *pvmdr1* codons 91, 976 and 1076.

This short exploratory chapter was the first to describe *pvmdr1* polymorphisms in the Philippines. The selection of samples and the success of amplification at *pvmdr1* codons 91, 976 and 1076 were presented in Figure 5.0. Fifty-nine *P. vivax* isolates from Mindanao were successfully genotyped at *pvmdr1* codon 91. Only the wild-type *pvmdr1* 91N allele was identified in these *P. vivax* isolates, which was confirmed twice

by PCR and sequencing assays. Two novel SNPs *pvmdr1* I86V and M87I were identified in one isolate each from South Cotabato Province. However, these two mutations were not confirmed due to poor amplification and sequencing in these two *P. vivax* isolates. It was likely that the *P. vivax* density in these two isolates were at the limit of detection of the PCR assays used to amplify *pvmdr1* codon 91 and there was a need to increase the sensitivity of the PCR assays designed to amplify these codons.

The *pvmdr1* codon 976 was successfully genotyped in 15.3% (9/59) *P. vivax* isolates from Mindanao while the *pvmdr1* codon 1076 was successfully genotyped in 5.1% (3/59) of *P. vivax* isolates from Mindanao. These were confirmed by repeat PCR and sequencing as previously described. Samples with negative PCR results were repeated twice but there was poor reproducibility and amplification in these two codons. Of the nine *P. vivax* isolates successfully genotyped at *pvmdr1* codon 976, 44.4% (4) have wild type *pvmdr1* 976Y while 55.6% (5) have the mutant *pvmdr1* 976F allele. The three *P. vivax* isolates successfully genotyped at *pvmdr1* codon 1076 all possessed the *pvmdr1* mutant allele 1076F. The *pvmdr1* haplotype for codons 91-976-1076 was constructed in three *P. vivax* isolates with complete data. PCR assays described in Chapter 3 confirmed that these three *P. vivax* isolates were single infections. The *pvmdr1* 91N-976**F**-1076**L** haplotype (mutated amino in bold font) was identified in two *P. vivax* isolates from Sarangani Province and from one *P. vivax* isolate from Tawi-Tawi Province.

Table 5.2. Single nucleotide polymorphisms in the *pvmdr1* gene of three *P. vivax* isolates from Mindanao, The Philippines with complete data.

Isolate	Origin	*Pvmdr1* alleles*		
		91	976	1076
P. vivax Salvador-1 (AY618622)	El Salvador, Central America	N	Y	F
Philippine isolates				
TKE 022	Sarangani Province	N	F	L
TKE 065	Sarangani Province	N	F	L
TT 531	Tawi-Tawi Province	N	F	L

*Alleles differing from the wild-type *P. vivax* Salvador-1 were highlighted in grey.

5.4. Discussion

Chloroquine resistance in *P. vivax* was suggested to be associated with polymorphisms in the *pvmdr1* gene (Brega et al., 2005, Suwanarusk et al., 2007, Suwanarusk et al., 2008) although genomic association studies were carried out to identify other loci (Orjuela-Sanchez et al., 2010). In the Philippines there were limited studies involving therapeutic efficacy of chloroquine against *P. vivax* malaria and this was the first attempt to describe polymorphisms in *pvmdr1* gene codons 91, 976 and 1076, which have been described in previous studies (Brega et al., 2005, Marfurt et al., 2008, Orjuela-Sanchez et al., 2009). These codons were homologous to *pfmdr1* codons 86, 1034 and 1042 where mutations were suggested to modulate levels of existing chloroquine resistance (Reed et al., 2000, Babiker et al., 2001).

None of the 59 *P. vivax* isolates has mutant allele at *pvmdr1* codon 91, which is homologous to the *pfmdr1* codon 86 where asparagine (N) to tyrosine (Y) mutation was associated with chloroquine resistance (Duraisingh et al., 1997, Pickard et al., 2003). Other studies did not find any mutation in *pvmdr1* codon 91 either, which is part of the predicted transmembrane domain 1 of the PVMDR1 protein (Brega et al.,

2005, Orjuela-Sanchez et al., 2009). The absence of mutation in the *pvmdr1* codon 91 of *P. vivax* isolates from Mindanao could be suggestive it was not selected by chloroquine. However, further studies would be needed to confirm this. Mutant alleles *pvmdr1* I86V and M87I were separately identified in each of the two *P. vivax* isolates from South Cotabato Province. Unfortunately, these two SNPs were not confirmed because the repeat amplifications of the fragment were not successful. These two *P. vivax* isolates were from South Cotabato where we expected the lowest parasite density in *P. vivax* positive individuals. Orjuela-Sanchez et al. (2009) identified unique *pvmdr1* N89S allele in the *pvmdr1* predicted transmembrane domain I (TMD) where codons 86, 87 and 91 were also found.

Genotyping of *pvmd1* codons 976 and 1076 was only successful in very few *P. vivax* isolates from Mindanao. This poor amplification could be attributed to insufficient optimisation of PCR assays targeting these two codons and the low density of parasites in the samples that would likely result to hit-or-miss of template DNA during PCR reactions. Further optimisation of the PCR conditions and the primers would have likely improved the sensitivity and specificity of these assays. With these existing limitations, *pvmdr1* codons 976 and 1076 were genotyped in nine and three isolates respectively. Five out of nine *P. vivax* isolates from Mindanao showed the mutant *pvmdr1* 976F allele, which has been previously proposed as an important marker of *P. vivax* resistance to chloroquine in other regions (Brega et al., 2005, Suwanarusk et al., 2007, Suwanarusk et al., 2008). The double mutant *pvmdr1* N**FL** haplotype (mutated amino acid in bold font) was identified in two isolates from Sarangani Province and one isolate from Tawi-Tawi Province with complete data. It would be interesting to mention that the double mutant *pvmdr1* N**FL** haplotype has been reported in in Indonesia, Thailand, and The Solomon Islands where chloroquine resistant *P. vivax* were already widespread (Brega et al., 2005, Tjitra et al., 2008). Chloroquine resistant *P. vivax* has not been reported in the Philippines (Baird et al., 1996a, World Health Organization, 2005) and this could be due to its long-standing partner drug primaquine. This effectively kills gametocytes and latent liver stages that might be activated in late in the course of infection when effective chloroquine concentration in blood was already suboptimal (Burgess and Bray, 1961, Warhurst, 1984). Given the

very small number of samples genotyped in this study, it would not be possible to infer whether the presence of mutant *pvmdr1* 976F allele and double mutant *pvmdr1* N**FL** haplotype were suggestive of chloroquine pressure in Mindanao. Further studies are needed to investigate the role of mutant *pvmdr1* 976F allele and mutant *pvmdr1* N**FL** haplotypes among *P. vivax* in Mindanao.

5.5. Conclusion

This exploratory study provided baseline data on *pvmdr1* polymorphisms in Mindanao, The Philippines that may be useful for future studies on *P. vivax* in the Philippines.

5.6. Limitations of the study

The *P. vivax* isolates used in this baseline study for characterizing *pvmdr1* in the Philippines were from subpatent infections where parasite densities were expected to be low and thus, the difficulty of repeating PCR amplification reactions. Further optimisation of PCR reactions targeting codons 976 and 1076 would have improved the sensitivity and specificity of the assays.

Chapter 6. Estimating malaria transmission using serological markers in low endemic provinces of Mindanao, The Philippines

6.1. Background

Malaria prevalence was substantially reduced in the Philippines as the country scaled up its control activities and adopted the ACT policy as the cornerstone treatment for *P. falciparum* malaria (World Health Organization, 2013). As a result several endemic provinces have experienced reduced transmission (Philippines National Malaria Program, 2014a). Evaluating malaria transmission in these provinces would be vital to plan malaria control and elimination to channel interventions where most needed (Carter et al., 2000). Malaria transmission intensity (MTI) is traditionally assessed using the entomological inoculation rate (EIR), which is the mean number of infectious mosquito bites per person per year. However, EIR has reduced sensitivity in low transmission settings because of small number and heterogeneity of infected mosquito vectors (Mbogo et al., 1995, Drakeley et al., 2003). Parasite prevalence can be proxy measure of transmission but its single time point estimate is unreliable to assess long-term transmission potential of an area (Drakeley et al., 2005). In addition, parasite prevalence is affected by fluctuating parasite density in peripheral blood, sensitivity of local diagnostic method used (O'Meara et al., 2007) and the very small number of symptomatic people in low transmission areas (Mosha et al., 2013). More so, estimation of *P. vivax* transmission can be further complicated by chance of relapses (Krotoski et al., 1982).

Immuno-epidemiological studies have explored the usefulness of antibody markers to *P. falciparum* and *P. vivax* apical membrane antigen 1 (AMA-1) and merozoite surface protein 1 (MSP-1) in estimating and monitoring changes in transmission to both species (Drakeley et al., 2005, Stewart et al., 2009, Bousema et al., 2010c, Cook et al., 2011, Cunha et al., 2014, Morais et al., 2006). AMA-1 is a type 1 integral membrane protein of merozoites with homologues found in all species of *Plasmodium* (Marshall et al., 1989, Peterson et al., 1989). The AMA-1 83-kDa prodomain found in the apical complex of schizont is cleaved to a 66-kDa mature protein for export to merozoite surface almost at the time of schizont rupture and erythrocyte invasion (Peterson et al., 1989, Narum and Thomas, 1994). It has been proposed that AMA-1 is essential to

erythrocyte invasion by facilitating formation of tight junction for passage of merozoite into the erythrocyte (Mitchell et al., 2004, Yap et al., 2014). People living in endemic areas develop natural antibodies to AMA-1 antigen (Udhayakumar et al., 2001, Wickramarachchi et al., 2006)

The *P. falciparum* MSP-1 precursor glycoprotein (195 kDa) is synthesised in schizonts and liver stages (Holder, 1988, Holder et al., 1992). This precursor is cleaved into fragments that form a non-covalently associated complex on the surface of the merozoite (Holder et al., 1987, McBride and Heidrich, 1987). Its C-terminal MSP-1_{42} fragment is sliced into MSP-1_{33}, which is shed off from the parasite (Blackman et al., 1991b, Blackman et al., 1991a). The smaller MSP-1_{19} fragment, which is left on the merozoite surface, is carried by the merozoite into the invaded erythrocyte suggesting that this fragment plays a role in erythrocyte invasion (McBride and Heidrich, 1987, Blackman et al., 1990). Since the merozoite is briefly exposed to the host's immune mechanisms prior to erythrocyte penetration, the MSP-1_{19} fragment becomes a potential target of invasion-blocking antibodies (O'Donnell et al., 2001, John et al., 2004). As the *P. falciparum* MSP-1_{19}, the *P. vivax* MSP-1_{19} also elicits natural antibodies in exposed individuals (Soares et al., 1999).

While frequent exposure is necessary to maintain non-sterile immunity to malaria infection, antibody responses to *P. falciparum* and *P. vivax* malaria can be maintained over time even without reinfection (Wipasa et al., 2010). The longevity of naturally acquired antibodies to AMA-1 and MSP-1_{19} antigens makes these surrogate markers useful in estimating malaria transmission (Rodrigues et al., 2003, Drakeley et al., 2005, Corran et al., 2007, Cunha et al., 2014). Antibodies to AMA-1 and MSP-1_{19} antigens represent cumulative exposure over time and are not subject to seasonality of vectors or fluctuating parasite densities particularly in low endemic areas (Wickramarachchi et al., 2006, Noor et al., 2008, Bousema et al., 2010c, Cook et al., 2010). Seroprevalence to AMA-1 or MSP-1_{19} antigens can be fitted into a simple reversible catalytic model to estimate malaria transmission (Williams and Dye, 1994, Drakeley et al., 2005, Corran et al., 2007).

As discussed by Corran et al. (2007) the simple reversible catalytic model assumes that in a given community, naïve individuals develop antibodies to a specific malaria antigen at the same rate at lambda (λ) per year throughout the period of exposure. This is the seroconversion rate. It also assumes that not all exposed individuals develop antibodies and those who do lose antibodies at the same rate at rho (ρ) per year (Corran et al., 2007). Parameter λ represents the force of infection, which can be estimate of malaria transmission intensity (Riley et al., 1996). Parameter ρ signifies persistence of antibodies in exposed individuals (Corran et al., 2007). In epidemiological studies estimates of λ for MSP-1_{19} and AMA-1 have shown good correlation with EIR (Drakeley et al., 2005, Corran et al., 2007). The choice of serological marker for malaria transmission is affected by immunogenicity and polymorphisms of chosen antigen (Drakeley et al., 2005). The highly immunogenic AMA-1 saturates fast among children in moderate transmission areas and thus, this is useful for estimation of transmission in low endemic areas (Corran et al., 2007, Stewart et al., 2009). MSP-1_{19} is less immunogenic and robust for use in moderate to high transmission areas. The estimated half-life of antibody to MSP-1_{19} is 50 years (Drakeley et al., 2005). Both *P. falciparum* and *P. vivax* AMA-1 and MSP-1_{19} antigens have limited diversity within species (Jongwutiwes et al., 1993, Kang and Long, 1995, Terheggen et al., 2014). In low transmission areas combining seroprevalence to AMA-1 and MSP-1_{19} increases the sensitivity in estimating transmission (Bousema et al., 2010c, Cook et al., 2010).

This chapter aimed to estimate malaria transmission in three endemic provinces of Mindanao, The Philippines using serological markers of exposure to *P. falciparum* and *P. vivax* transmission.

6.2. Methods

Ethics statement

The LSHTM Ethics Committee (reference No. 5712) and the National Ethics Committee of the Philippines approved this research involving human participants in 2010.

Study sites and sample collection

This study was conducted in three malaria endemic provinces of Mindanao, The Philippines. Sarangani Province and South Cotabato Province are both located in Southern Mindanao mainland while Tawi-Tawi is an island province at the southernmost tip of the Philippines. Sarangani Province is a second-class province based on income and it has seven municipalities and 141 villages. The province is typically hilly and mountainous with numerous creeks and rivers. It bounded at the east by the Alip Range and at the north by the Daguma Range, which separates two municipalities of Sarangani from South Cotabato (de Jesus et al., 2001). Rainfall is more or less evenly distributed throughout the year and is heavier from May to October. It is among the poorest provinces in the country with the B'laan as the dominant tribe (Province of Sarangani, 2010, Philippine Statistics Authority, 2014). South Cotabato is a first class province composed of two cities, 10 municipalities and 255 villages. This province has upland lakes, plateaus, dormant volcanoes and a mountain range that separates it from one side of Sarangani Province. Rainfall is also nearly evenly distributed throughout the year in South Cotabato with most occurrences from June to August. The dominant tribe is T'boli (South Cotabato Provincial Health Office, 2008, Philippine Statistics Authority, 2014). Tawi-Tawi is a third class province of 106 islands and islets. It has 11 municipalities and 203 villages (Philippine Statistics Authority, 2014). In this province rainfall is more abundant from August to November. Sarangani and Tawi-Tawi have stable malaria while South Cotabato has unstable malaria transmission (Philippines National Malaria Program, 2014f). From these three provinces, a total of 2,068 samples were collected from consenting participants across all ages during cross-sectional surveys. These samples were kept dried in ambient temperature and transported to the LSHTM, United Kingdom for serological analyses.

Elution and storage of sera

Serum from 6mm dried blood spot on Whatman 3MM filter paper was eluted using 1mL IX PBS containing 0.5% saponin. Assuming that each 6 mm blood spot contained 10 µL of whole blood, then whole blood was diluted 1:100 equivalent to 1:200 serum dilutions (Corran et al., 2008). Sera were stored at -40C prior to serological assays.

Screening for antibodies to AMA-1 and MSP-1 antigens

Samples were tested for presence of human antibodies to recombinant PfAMA-1 antigen (3D7), PfMSP-1_{19} (Wellcome genotype), PvAMA-1 (Salvador genotype) or PvMSP-1_{19} (Belem genotype) using indirect enzyme-linked immunosorbent assay (ELISA). The production of these recombinant antigens and the determination of the working dilution of each antigen using antigen titre ELISA were developed in the Drakeley Laboratory and were outside the scope of this study. Each antigen tested was coated on a 96-well high absorbance plate (Immunolon 4HBX, Thermo) at a dilution previously determined using antigen titration ELISA and stored overnight at overnight at 4°C. The plates were washed three times in 1X Phosphate Buffered Saline (PBS) solution containing 0.5% Tween 20 (PBS/T), dried and blocked with 150 µL of 1% skim milk powder in PBS/T. After three hours the plates were washed in PBS/T, dried and blocked with 1% skim milk. When screening for antibodies to AMA-1, duplicates of 5 µL serum samples were added to 45 µL blocking solution to achieve 1:2,000 serum dilution. When screening for antibodies to MSP-1_{19}, duplicates of 10 µL serum samples were added to 40 µL blocking solution to obtain a 1:1,000 final serum dilution. It was at these final dilutions that screened antibodies were often detected at an optical density (OD) above that of the control without excluding sera containing low concentration of target antibodies (Drakeley et al., 2005). Standard African hyperimmune plasma pool added in duplicate series was used as positive control, which was diluted in six four-fold serial dilutions. Two duplicates of negative control or blank containing 50 µL blocking solution were present in each plate. The processed plates were incubated overnight at 4°C. These were washed five times with PBS/T and

dried before adding 50 µL horseradish peroxidase-conjugated rabbit anti-human immunoglobulin G (Dako, Roskilde, Denmark) previously diluted at 1:5,000. After three hours in room temperature, the plates were washed five times with PBS/T.

The indirect ELISA using OPD substrate was used to screen samples from Sarangani Province collected in 2010. Samples collected from Tawi-Tawi and South Cotabato in 2012 and 2013 were screened using the same indirect ELISA but the substrate used was tetramethylbenzidine (TMB) (Sigma-Aldrich) as a result of transitioning to less toxic substrate. When using 100 µL TMB as substrate, the anti-human IgG was diluted to 1:15,000. The reaction was stopped by adding 50 µL of 0.2 M H_2SO_4. The Drakeley Laboratory in the LSHTM has validated this indirect ELISA using TMB substrate against the OPD-based assay to yield comparable results as the latter before being adopted for use in this study.

Data analyses

Samples were excluded from analysis if there were 20% variation between duplicate optical density (OD) values. Using a Microsoft Excel with solver add-in the duplicate raw OD values of samples were averaged, corrected and normalised against the positive controls in each plate using a three-sigmoid parameter model (Corran et al., 2008). The corrected OD would have been the OD obtained had the plasma been more dilute while the normalised OD was the adjusted value to account for variations within and between plates (Corran et al., 2008). The distribution of the normalised OD values was fitted into a finite mixture model using maximum likelihood methods, which divided the samples into seronegative and seropositive Gaussian distributions (Stewart et al., 2009). For each antigen the cut-off for seropositivity was calculated as the mean of the normalised OD plus 3 standard deviations of the seronegative population's Gaussian distribution (Cook et al., 2010). The normalised OD values were compared to calculated cut-off to classify samples as seropositive or seronegative. Results were reported as seroprevalence or the proportion of individuals with antibodies to the

antigen tested. In this study seroprevalence data to AMA-1 and MSP-1_{19} were combined for *P.* falciparum and *P. vivax.*

Only participants who provided their age during the surveys and who were successfully screened for antibodies to AMA-1 and MSP-1_{19} antigens of *P. falciparum* or *P. vivax* were included in estimating seroprevalence and malaria transmission. Age seroprevalence data were fitted into a simple reversible catalytic model using maximum likelihood methods assuming binomial error distribution (Corran et al., 2007, Williams and Dye, 1994). The model generated age seroprevalence, which was used to calculate seroconversion (λ) and seroreversion (ρ) rates. Parameters λ and ρ were assumed to be uniform in all age groups coming from a particular place, in this case a province in Mindanao, at time *t.* Although parameter ρ could be constrained to improve the fit of the data (Drakeley et al., 2005), the model was allowed to estimate parameter ρ in this study. If the seroprevalence plot was suggestive that λ was not uniform across, then models allowing for two λs were used to predict the time (year) when the change in transmission intensity most likely occurred. This was only accepted if the fit was significant compared to the model with single seroconversion rate (Cook et al., 2011). All statistical analyses were carried out using Stata version 13 (Statacorp, Texas).

6.3. Results

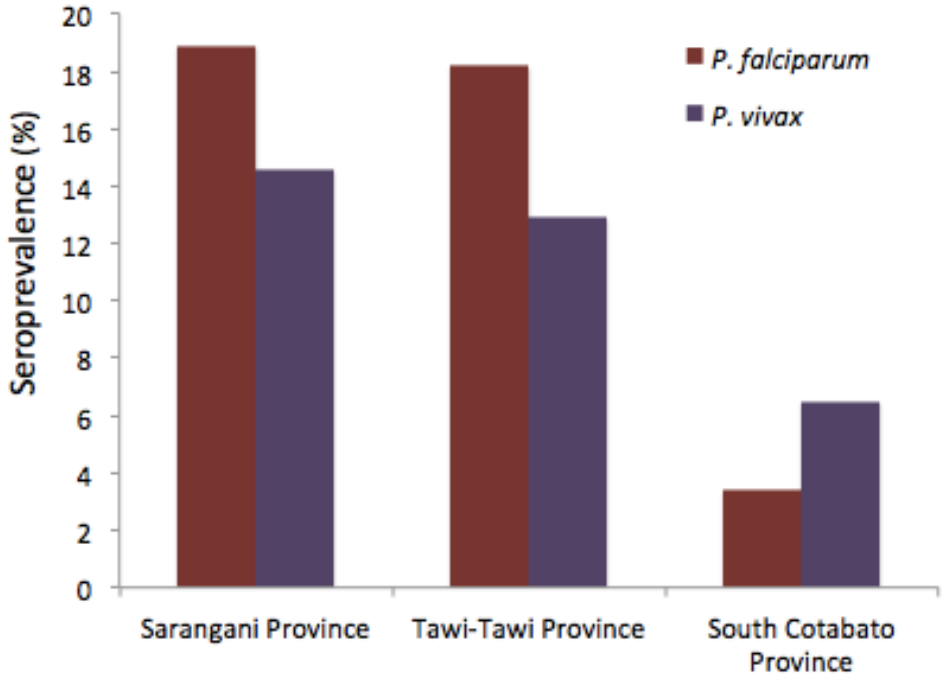


Figure 6.1. Seroprevalence to *P. falciparum* and *P. vivax* in three provinces of Mindanao, The Philippines estimated by combining seroprevalence to AMA-1 and MSP-1_{19} antigens for each species.

In this study where malaria transmission was expected to be low, seroprevalence to *P. falciparum* and *P. vivax* was estimated by combining seroprevalence to AMA-1 and MSP-1_{19} antigens for each species. In Sarangani Province seroprevalence to *P. falciparum* and *P. vivax* was successfully determined in 88.0% (826/939) and 74.3% (698/939) of participants respectively. In this province exposure to *P. falciparum* infection (18.9%) was found to be significantly higher than exposure to *P. vivax* infection (14.6%) at p=0.026 (Figure 6.1). Seroprevalence to *P. falciparum* and *P. vivax* was successfully determined in 96.2% (1,047/1,088) and 94.4% (1,027/1,088) of participants respectively in Tawi-Tawi Province. In this province exposure to *P. falciparum* infection (18.2%) was higher than exposure to *P. vivax* infection (12.9%) at p=0.001. Meanwhile in South Cotabato, 18.2% (109/600) of participants were not included in the analyses because of missing age data. Among those with complete age data, 96.0% (473/492) and 96.5% (475/492) have seroprevalence data for *P.*

falciparum and *P. vivax* respectively. Contrary to seroprevalence data in Sarangani Province and Tawi-Tawi Province, exposure to *P. falciparum* (3.4%) was significantly lower than exposure to *P. vivax* (6.5%) in South Cotabato Province at p=0.028 (Table 6.1).

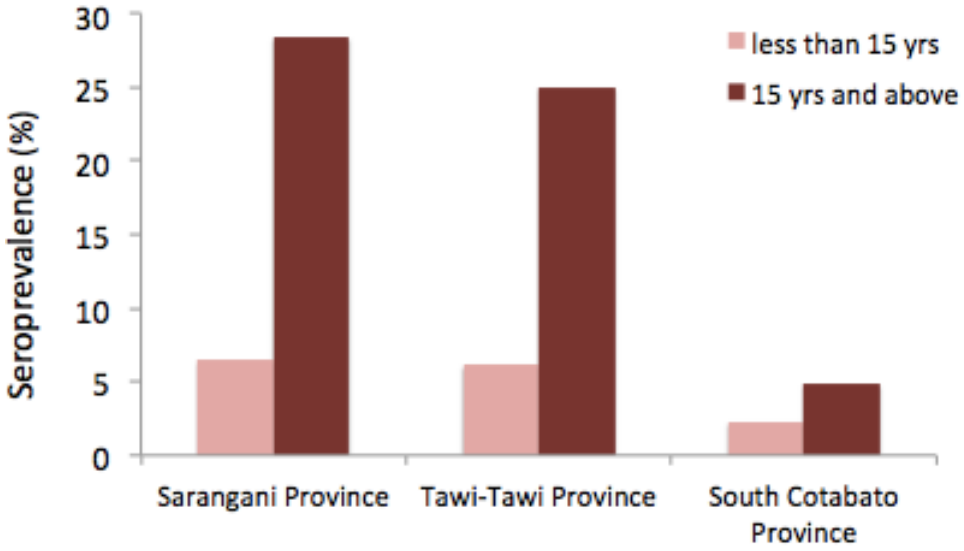


Figure 6.2. Seroprevalence to *P. falciparum* by age group in three endemic provinces in Mindanao, The Philippines. Seroprevalence to *P. falciparum* was estimated by combining seroprevalence to *P. falciparum* AMA-1 and MSP-1_{19} antigens.

As expected seroprevalence to *P. falciparum* was higher in adults (≥15 years old) than in children (<15 years old) in the three provinces surveyed (Figure 6.2). In Sarangani Province 6.4% (23/358) and 28.4% (133/468) of children (<15 years old) and adults (≥15 years old) were seropositive for *P. falciparum* respectively. In Tawi-Tawi Province 6.1% (23/375) of children and 28.4% (168/672) of adults were seropositive for *P. falciparum*.

Seroprevalence to *P. falciparum* was less than 5% for both age groups in South Cotabato Province whereby only 2.3% (6/265) of children and 4.8% (10/208) of adults

showed seropositivity for *P. falciparum*. Seroprevalence between the two age groups was significant in Sarangani Province and Tawi-Tawi Province at p=0.000 each. In South Cotabato seroprevalence between two age groups was not significant at p=0.129.

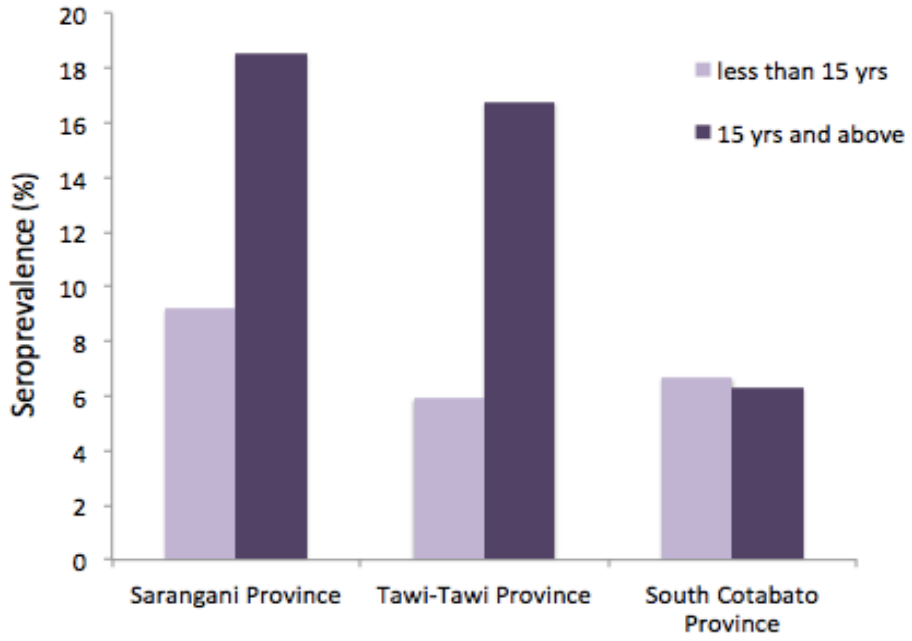


Figure 6.3. Seroprevalence to *P. vivax* by age group in three endemic provinces in Mindanao, The Philippines. Seroprevalence to *P. vivax* was estimated by combining seroprevalence to *P. vivax* AMA-1 and MSP-1_{19} antigens.

Seroprevalence to *P. vivax* was higher in adults (≥ 15 years) than in children (<15 years) in Sarangani Province and Tawi-Tawi Province (Figure 6.3). In Sarangani Province 9.2% (27/293) of children and 18.5% (75/405) of adults were seropositive to *P. vivax* while in Tawi-Tawi Province, 5.9% (22/371) of children and 16.8% (110/656) of adults were seropositive to *P. vivax*. The estimated seroprevalence between age groups was significant in Sarangani Province (p=0.001) and Tawi-Tawi Province (p=0.000). Against expectations there was no significant difference in seropositivity to *P. vivax* between

children (6.7%, 18/269) and adult (6.3%, 13/206) participants in South Cotabato Province at p=0.868.

There were six children who were five years old and below at the time of survey in Sarangani Province in 2010 that were found seropositive to *P. falciparum* and *P. vivax*. Two children of the same age group were seropositive to *P. vivax* in South Cotabato Province. In Tawi-Tawi Province there were two children of the same age group who were seropositive to *P. falciparum*. During the surveys in Sarangani Province in 2010, none of the participants complained having fever "*hilanat*" or chills "*ginakurog*". None of the participants in Tawi-Tawi Province complained of fever "*mapasu in baran*" or chills "*tyandug*" during the surveys in 2012. Similarly, none of the survey participants in South Cotabato complained of fever "*kini*" or chills "*kenkel*" during the surveys in 2013. The malaria field teams who conducted the surveys were very familiar with these terms in local dialects in each province. However, the questionnaire used in the three provinces was not able to capture recent fever episodes in each participant.

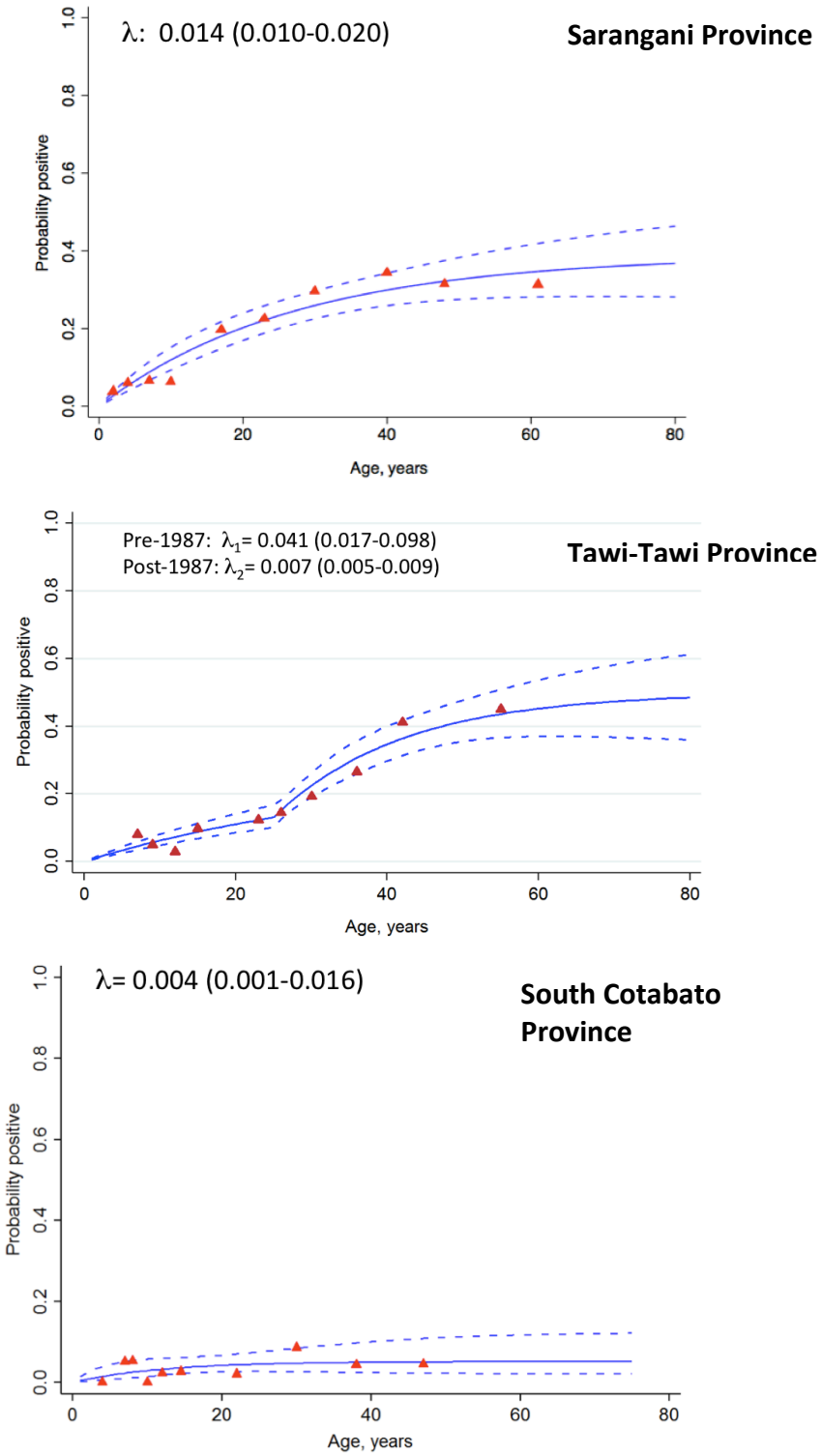
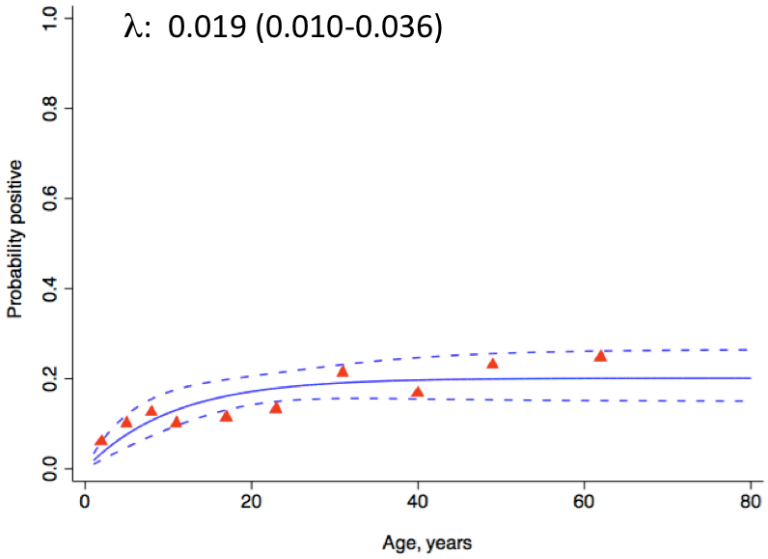


Figure 6.4. Age seroprevalence plots for *P. falciparum* in Sarangani Province in 2010, in Tawi-Tawi Province in 2012 and in South Cotabato in 2013. Observed age seroprevalence are represented by red triangles. The maximum likelihood plot and 95% confidence interval are represented by unbroken and broken lines respectively.

The seroconversion rate (λ) for *P. falciparum* estimated by combining seroprevalence to *P. falciparum* AMA-1 and MSP-1_{19} antigens was low in the three provinces surveyed (Figure 6.4). In Sarangani Province the estimated SCR or λ was 0.014 (95% CI 0.010-0.020), which could be interpreted as 14 new *P. falciparum* infections per 1,000 people per year. This was the force of infection. In Tawi-Tawi Province visual inspection of the seroprevalence plot generated from a model estimating a single λ suggested that malaria transmission intensity in this province changed over time. This prompted the use of a model allowing two forces of infection to estimate changes in *P. falciparum* transmission in Tawi-Tawi Province. The resulting age seroprevalence plot shown in Figure 6.4 estimated that a change occurred in *P. falciparum* transmission intensity in Tawi-Tawi Province at about 25 years before the time of survey. This suggested the SCR for *P. falciparum* was 0.041 (95% CI 0.017-0.098) in Tawi-Tawi Province 25 years before the survey (at about year 1987) but this significantly dropped by almost six-fold (SCR 0.007, 95% CI 0.005-0.009) after 1987. In South Cotabato Province, the seroprevalence plot suggested that *P. falciparum* exposure was almost uniformly distributed across different age groups. It has the lowest estimated SCR (0.004, 95% CI 0.001-0.016) for *P. falciparum* transmission suggesting that the force of *P. falciparum* infection was the lowest in this province.

Estimated SCRs for *P. vivax* were low in Sarangani Province and Tawi-Tawi Province (Figure 6.5). In Sarangani Province the estimated SCR for *P. vivax* was 0.019 (95% CI 0.010-0.036), which could be interpreted as 19 new *P. vivax* infections per 1,000 people per year. This was higher than the estimated force of infection for *P. falciparum* in the Sarangani Province. In Tawi-Tawi Province the estimated SCR for *P. vivax* was 0.006 (95% CI 0.005-0.007) (Figure 6.5), which was lower than the SCR for *P. falciparum* in the province. The model was not able to generate an age seroprevalence plot for *P. vivax* or calculate parameters λ and ρ for *P. vivax* transmission in South Cotabato. This suggested that in 2013 *P. vivax* exposure was uniform across all ages in South Cotabato.

Sarangani Province



Tawi-Tawi Province

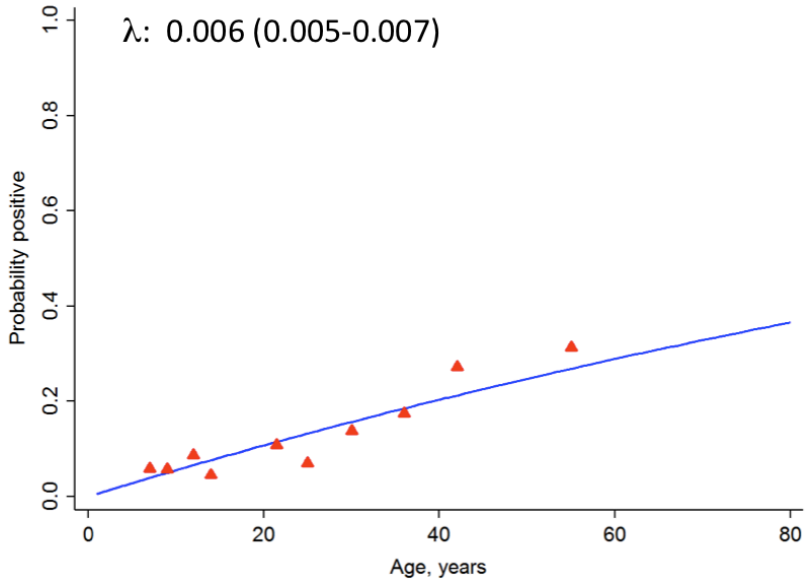


Figure 6.5. Age seroprevalence plots for *P. vivax* in Sarangani Province in 2010 and in Tawi-Tawi Province in 2012. Observed age seroprevalence are represented by red triangles. The maximum likelihood plot and 95% confidence interval are represented by unbroken and broken lines respectively. There was no age seroprevalence plot for *P. vivax* generated for South Cotabato Province.

6.4. Discussion

The Philippine government aimed to eliminate malaria in its 53 endemic provinces by 2020 (Department of Health Philippines, 2011). This would require strategic planning and interventions based on current malaria transmission intensity (Carter et al., 2000). Sarangani Province and Tawi-Tawi Province have stable malaria transmission in 2010 and 2012 respectively (Department of Health Philippines, 2011) while South Cotabato has unstable malaria transmission in 2013 (Philippines National Malaria Program, 2014a). Yet, microscopy remained as the operational standard for malaria diagnosis in the Philippines (Department of Health Philippines, 2010). With the decline of malaria in the Philippines in the last decade (World Health Organization, 2013) and the reduced sensitivity of microscopy in low endemic settings (Harris et al., 2010, Mosha et al., 2013), parasite prevalence would be poor measure of malaria transmission in the study provinces.

In this study malaria transmission in three endemic provinces of Mindanao was estimated by combining seroprevalence to AMA-1 and MSP-1_{19} antigens. Sarangani and Tawi-Tawi have stable malaria transmission at the time of the surveys (Department of Health Philippines, 2011, Philippines National Malaria Program, 2013). In these provinces it was not surprising that in *P. falciparum* and *P. vivax* seroprevalence was higher than parasite prevalence by PCR. Seroprevalence represented period prevalence as a result of repeated exposure to antigen tested while parasite prevalence only portrayed a single point in time (Cook et al., 2010). Similar results were obtained in low endemic areas where microscopy and RDT were insensitive in detecting asymptomatic people (Bousema et al., 2010b, Cook et al., 2011). Seroprevalence to *P. falciparum* was higher than seroprevalence to *P. vivax* in Sarangani Province and Tawi-Tawi Province, which suggested that these provinces experienced higher exposure to *P. falciparum*. This was expected since *P. falciparum* has been the dominant circulating parasite in the Philippines (Cabrera and Arambulo, 1977, Belizario et al., 1997, World Health Organization, 2005, World Health Organization, 2013). In South Cotabato malaria prevalence was very low since 2001

(Philippines National Malaria Program, 2014a, Philippines National Malaria Program, 2013). When this study was conducted in early 2013, there was no reported malaria case in South Cotabato Province (Philippines National Malaria Program, 2013) although there were unconfirmed reports in remote villages of Lake Sebu and T'boli. Parasite prevalence by PCR and seroprevalence data obtained from South Cotabato Province suggested that malaria was still occurring in remote communities in this province at levels below the detection of the RDT. Our data showed that there was no difference between parasite prevalence by PCR and seroprevalence. In addition, seroprevalence to both species was lowest in South Cotabato Province compared to Sarangani Province and Tawi-Tawi Province. It was likely that herd immunity to malaria has dropped with infrequent exposure and very low incidence of malaria for several years in South Cotabato Province (Ghani et al., 2009, Cook et al., 2010, Wipasa et al., 2010). While there was no detectable difference in parasite prevalence by PCR between children and adults in South Cotabato, seroprevalence to *P. falciparum* (3.4%, 16/473) was significantly lower than seroprevalence to *P. vivax* (6.5%, 31/475) at p=0.026. This suggested that South Cotabato Province where malaria transmission was already very low, serological markers provided extra sensitivity over parasite prevalence to detect and examine differences in *P. falciparum* and *P. vivax* transmission.

Seroprevalence data in Sarangani Province and Tawi-Tawi Province showed age-dependent acquisition of antibodies to the antigens tested (Drakeley et al., 2005, Corran et al., 2007). These antibody responses could persist for months to years even with infrequent infection (Morais et al., 2006, Wipasa et al., 2010). This suggested that seropositivity to both species among adults would not necessarily imply recent exposure (Bousema et al., 2010b). Seropositivity in children could be used as indicators of an existing focus of malaria (Bousema et al., 2010b, Cook et al., 2010) and this could be used to examine heterogeneity of malaria transmission (Cook et al., 2011). In this study there were children aged 5 years and below who were seropositive to *P. falciparum* and *P. vivax* in Sarangani Province, to *P. falciparum* in Tawi-Tawi Province, and to *P.* vivax in South Cotabato Province. These children might be from communities with ongoing low-level transmission. It has been previously mentioned that the questionnaire used in this study was not able to include recent fever episodes and

treatments of participants. As discussed in Chapter 3 these children might have previous fever episodes but this information was not included in this study. These children might eventually develop clinical symptoms depending on parasite densities in their peripheral blood. Age would be an important factor in acquisition of partial immunity to clinical malaria and parasite carriage (Filipe et al., 2007, Males et al., 2008). Infected people in high transmission areas would be asymptomatic because population immunity would be maintained by frequent exposure (Daubersies et al., 1996, Trape et al., 1994) but in low transmission settings people would have lost acquired immunity and develop clinical disease. However, asymptomatic malaria infections have been reported in the recent years in low endemic settings (Belizario et al., 1997, Alves et al., 2002, Harris et al., 2010). This suggested that malaria transmission persisted at low levels and this was responsible for maintaining immunity in the population. In South Cotabato there was no difference in seroprevalence to *P. falciparum* and *P. vivax* between children (<15 years) and adults (15 years and above) suggesting that exposure of people to both species of *Plasmodium* was uniformly low across all ages (Cook et al., 2010).

Although recent fever episodes were not included in the information surveyed, this study was able to describe malaria transmission intensity in the three provinces surveyed using age-dependent antibody responses. The simple reversible catalytic model allowed estimation of SCR per province, which corresponded to the force of infection (Riley et al., 1996, Drakeley et al., 2005). SCRs for *P. falciparum* and *P. vivax* suggested that malaria transmission was low at the time of the survey. It was only in Tawi-Tawi Province where the model depicted a change in *P. falciparum* transmission intensity approximately 25 years before the survey. It was not known what event directly influenced this significant change in *P. falciparum* transmission in Tawi-Tawi Province. It could be due to the decentralisation of health systems in the Philippines in the early 1980s (Espino et al., 2004) or major changes in the local and national political systems of the country following the People Power Revolution in 1986. It could be brought about by other factors such as improvement in educational system or malaria control measures in the province. These assumptions would require further investigation in a separate study. SCR estimate for *P. falciparum* was lowest in South

Cotabato suggesting that the province was gaining ground towards *P. falciparum* elimination. It was more likely that people were already losing antibodies to *P. falciparum* during the years of sustained control interventions (Ghani et al., 2009). SCR for *P. vivax* was three-fold higher in Sarangani Province relative to Tawi-Tawi Province. The simple reversible catalytic model was not able to estimate SCR for *P. vivax* in South Cotabato suggesting that exposure was uniform across all ages.

6.5. Conclusion

Combining serological responses to AMA-1 and MSP-1_{19} antigens allowed us to determine that malaria transmission in Sarangani Province and Tawi-Tawi Province was already low. We also found that in South Cotabato Province malaria transmission was still ongoing despite absence of reported malaria cases by microscopy since 2010. In these provinces combining parasitological data by PCR and serological data could enhance potential to evaluate and target focal areas still undergoing transmission. It seemed that each province has the biological feasibility of eliminating malaria particularly South Cotabato Province. However, the risk posed by asymptomatic *P. falciparum* and *P. vivax* infections, remote focal areas of transmission and population movement between endemic provinces must be further explored.

Chapter 7. Summary of findings, discussion, limitations & future work

7.1 Background

There is limited information on circulating *Plasmodium* species, malaria transmission and genetic background of *P. falciparum* and *P. vivax* in many malaria-endemic provinces in the Philippines. In this study, estimates of *P. falciparum* and *P. vivax* infections in three provinces were determined in cross-sectional surveys. *P. falciparum* and *P. vivax* isolates were genotyped where possible with key genes *crt* and *mdr1*, and cross-sectional serology was done to provide insight on immunoepidemiology.

7.2. Summary of Findings

Continuous subpatent reservoirs of malaria. The epidemiology of malaria in Sarangani Province, South Cotabato Province and Tawi-Tawi Province was described in Chapter 3. Consistent with previous reports in the Philippines, the main *Plasmodium* species identified in these provinces were *P. falciparum* and *P. vivax*. There was one clinical case of imported *P. malariae* in South Cotabato Province and there was no human infection with *P. ovale* spp. or zoonotic *P. knowlesi*. Against expectations, individuals with *P. falciparum* and *P. vivax* infections diagnosed in cross-sectional surveys in the three provinces have subpatent infections. In addition, subpatent infections of *P. falciparum* and *P. vivax* were diagnosed in South Cotabato Province where malaria cases by microscopy have not been reported since 2010. Since all malaria infections were subpatent and therefore with generally low parasite density, there were discrepancies between using RDT FalciVax™ and PCR diagnoses in Sarangani Province and South Cotabato Province, and between using microscopy and PCR diagnoses in Tawi-Tawi Province. Even PCR diagnosis was subject to poor reproducibility.

Reduced malaria transmission. Chapter 6 provided insight on the immunoepidemiology and transmission of malaria in Sarangani Province, South Cotabato Province and Tawi-Tawi Province. Seroconversion rate (SCR) estimates for *P. falciparum* and *P. vivax* suggested low-level transmission in Sarangani Province and

Tawi-Tawi Province. Presence of very low *P. falciparum* and *P. vivax* transmission in South Cotabato Province was not expected since this province has declared absence of malaria cases since 2010. Age-specific seroprevalence plots suggested two forces of infection in the history of *P. falciparum* and *P. vivax* malaria in Tawi-Tawi Province but the statistical power to detect that such differences in forces of infection existed was not sufficient based on the number of people sampled in each province.

Circulating *pfmdr1* and *pfcrt* haplotypes. The *pfcrt* CVIET and SVMNT haplotypes found in *P. falciparum* isolates from Mindanao suggested chloroquine and amodiaquine pressure respectively. The *pfcrt* A144T and L160Y mutant alleles previously reported in the Philippines were not found in *P. falciparum* isolates from Mindanao suggesting that other mutations could be found elsewhere in the *pfcrt* gene to compensate for absence of the *pfcrt* A220S mutation in *P. falciparum* with mutant *pfcrt* 76T mutant allele. The low frequency of mutant *pfmdr1* haplotypes might be due to withdrawal of chloroquine and its replacement with artemether-lumefantrine for treatment of *P. falciparum* infection in 2009 (Department of Health Philippines, 2010) but further studies are needed to demonstrate this. The presence of *pfmdr1* NFSND haplotype, which was associated with prolonged *P. falciparum* survival in artemether-lumefantrine (AL) (Dokomajilar et al., 2006, Happi et al., 2009), might affect the sensitivities of parasites to AL in Sarangani Province and Tawi-Tawi Province.

Circulating *pvmdr1* alleles. Polymorphisms in the *pvmdr1* gene of selected *P. vivax* isolates from Mindanao showed that mutant *pvmdr1* Y976F and F1076L alleles were present few *P. vivax* isolates from Sarangani Province and Tawi-Tawi Province. Further studies are needed to understand the implications of these mutant alleles to the use of chloroquine and primaquine as first-line treatment for *P. vivax* infection.

7.3. Discussion

In areas where malaria is endemic, repeated exposure to malaria leads to development of acquired immunity that protects people from developing clinical malaria (Druilhe and Perignon, 1997, Staalsoe and Hviid, 1998, Males et al., 2008). When transmission is reduced, a degree of herd immunity is lost and those with malaria may become symptomatic (Ghani et al., 2009). We envisaged a similar situation in the study provinces where malaria cases were reduced in the last 5 years (Philippines National Malaria Program, 2013). Contrary to this expectation, none of the participants from Sarangani Province, South Cotabato Province and Tawi-Tawi Province diagnosed with *P. falciparum* and *P. vivax* malaria presented with clinical malaria; these findings are similar to those of Harris et al. (2010) in a province of The Solomon Islands with very low malaria transmission. These subpatent *P. falciparum* and *P. vivax* infections constituted important parasite reservoirs responsible for perpetuating transmission and maintaining herd immunity at very low levels (Druilhe and Perignon, 1997, Bousema et al., 2004, Van den Eede et al., 2011). Without sensitive molecular diagnostic tool, these subpatent infections would remain undetected (Sturrock et al., 2013) . As expected in the Philippines (World Health Organization, 2013), the prevalence of *P. falciparum* was higher than that of *P. vivax* in Sarangani Province and Tawi-Tawi Province. Contrary to this expectation, the prevalence of *P. vivax* was higher than *P. falciparum* in South Cotabato Province where indigenous malaria cases identified by microscopy have not been reported since 2010. This suggests that *P. vivax* was more difficult to control and eliminate than *P. falciparum* (Looareesuwan et al., 1987) such that successful control measures against *P. falciparum* may not necessarily ensure reduction of *P. vivax* burden.

There were discrepancies in diagnosis of malaria infection using microscopy and antigen detection because of low frequency of infected humans and because parasite density, was at the limit of detection of either method. Thus, the conventional parasitological measure “parasite rate” was not sensitive to assess malaria transmission in the study provinces. In these settings, serological markers were used to

estimate transmission since antibodies reflect cumulative exposure over time and are not affected by seasonality of vectors or low number of infected humans with detectable malaria parasites in their peripheral blood (Drakeley et al., 2005, Corran et al., 2007, Stewart et al., 2009). Overall seroprevalence to *P. falciparum* and *P. vivax* AMA-1 and MSP-1_{19} antigens were low in the study provinces such that combining seroprevalence to one or both antigens in each *Plasmodium* species provided additional sensitivity in assessing transmission as done in other low endemic settings (Bousema et al., 2010c, Cook et al., 2010). Using a simple reversible catalytic model (Corran et al., 2007), the estimated seroconversion rates (SCR) for *P. falciparum* and *P. vivax* transmission were very low in the provinces surveyed. These evidences supported findings in Chapter 3 that malaria transmission is still continuous at low levels in Sarangani Province, Tawi-Tawi Province, and especially noteworthy, in South Cotabato Province where malaria cases by microscopy have not been reported since 2010. The seroprevalence data further suggested that malaria was heterogeneous within the provinces surveyed. This could be due to variable impact of malaria control measures (Cook et al., 2011) particularly in areas with poor geographic access to diagnosis and treatment. Such remote populations could maintain reservoirs of malaria infection (Bousema et al., 2010a, Bousema et al., 2012, Sturrock et al., 2013). The implications of continuous low-level malaria transmission as subpatent infections should be further explored when planning malaria elimination in these provinces. One essential drawback in the use serology was the longevity of anti-malarial antibodies in the population such that it is essential to monitor seroconversion in young children, which is tedious and will require a large sample size, or do a direct parasite test using a molecular tool that can be brought to the field to test for presence of malaria infection.

Of particular concern in Sarangani Province, South Cotabato Province and Tawi-Tawi Province was the presence of subpatent *P. falciparum* and *P. vivax* infections carrying alleles in key genes that might influence parasite sensitivities to current antimalarial drugs in the Philippines. It is important to prolong the usefulness of artemether-lumefantrine in treating uncomplicated *P. falciparum* malaria in the Philippines because it has been recognised in other endemic settings that artemether-

lumefantrine select certain alleles in the *pfcrt* and *pfmdr1* genes (Sisowath et al., 2005, Humphreys et al., 2007, Sisowath et al., 2009, Gadalla et al., 2011, Henriques et al., 2014), which were also associated with chloroquine and amodiaquine resistance (Djimde et al., 2001, Ochong et al., 2003, Mita et al., 2006, Beshir et al., 2010, Gadalla et al., 2015). Although there is no definite molecular marker for *P. vivax* resistance to 4-aminoquinolines, previous studies associated the *pvmdr1* mutant Y976F allele with reduced *P. vivax* sensitivity to chloroquine and amodiaquine treatment (Brega et al., 2005, Suwanarusk et al., 2007, Marfurt et al., 2008). Further studies with larger sample sizes are needed to understand that implications of the *pvmdr1* mutant Y976F allele in Sarangani Province and Tawi-Tawi Province.

It should be noted that the collection period and collection months differed per province as these were affected by availability of funding, coordination with local collaborators and the peace and order situation in the study sites. These differences might impact the comparative results since the endemicity of malaria in these provinces were expected to vary from 2010 to 2013. Thus, the results would be suggestive of malaria endemicity in the study site for that particular time point in the survey.

7.4. Limitations of the Study

Several factors were identified to have potential impact on the study:

1. **Sample size**. This study calculated a sample size of 300 per municipality to detect at least one person with malaria by PCR. Since the transmission was low in the provinces surveyed, large sample sizes would have given higher statistical power to detect significant differences in malaria epidemiology and transmission. Population surveys were not possible since funding for fieldwork was limited.

2. **Questionnaire**. The questionnaire used in this study was translated from its English version into versions in local dialects, which were reviewed by researchers and local collaborators before use in the field. However, pre-testing and back translating the questionnaire would have identified information lacking such as recent fever episodes and axillary temperature. It would have improved phrasing questions to gather information such as age data among others.
3. **Age distribution**. Age group was not equally represented among participants and this affected the age-seroprevalence plot for estimating malaria transmission in Chapter 6. Not all participants were willing to queue and wait, especially mothers with small children and people who lived in distant communities from the common place of survey. This would have been improved by inviting more participants but survey team members were not allowed to stay in remote communities later than 4pm or stay overnight for safety reasons.
4. **Missing age data**. Exact age in years is important in estimating force of malaria infection in Chapter 6. Several adult participants in South Cotabato did not provide their exact age in years and there was no other information in the questionnaire that could provide clue to their biological age. This could have been obtained by asking these participants if they were born on the same year as an important local or national event, *i.e.* start of World War II, declaration of Philippine independence, Martial Law in the Philippines, etc.
5. **Low parasite density**. There were discrepancies when using microscopy, antigen detection and gene amplification with PCR in diagnosing subpatent malaria infections. It was very likely that the parasite densities in these samples were at the limit of detection of the diagnostic methods used. Even PCR assays have poor reproducibility due to low parasite density. Further optimisation of PCR assays designed in this study would have improved sensitivity. A diagnostic tool with sensitivity and specificity comparable to PCR should be tested in these low endemic provinces. In addition, this study used Chelex method in extracting DNA for speciation PCR and the QIAGEN kit method was used for extracting DNA used in molecular genotyping. Both extraction methods still

gave variable results in PCR suggesting that DNA extraction methods for subpatent infections should be re-evaluated.

Given sufficient funding opportunities, this study would benefit from two-time point population surveys involving large sample sizes since malaria transmission was already low. This would provide higher power to detect significant differences in parasite prevalence, effects of population factors such as age in exposure to *P. falciparum* and *P. vivax* and provide opportunity to observe change in parasite prevalence at two time points. It would also benefit when stringent methods were used in selection of study sites although in Mindanao, this would be largely dependent on peace-and-order situation on ground. There should have been criteria for selection of participants so all ages would be equally represented and questionnaire survey should be validated before use to capture sufficient epidemiologic information in the study sites. Lastly, further optimisation of PCR assays would have given salient information in key genes characterised in this study.

Despite these limitations, this study provided important baseline information in the epidemiology of malaria in Sarangani Province, South Cotabato Province and Tawi-Tawi Province. These data could be useful for local and national malaria programs in planning malaria elimination in this region.

7.5 Future work

These areas should be further explored in Mindanao based on the outputs of this study:

Pursuing malaria control and elimination at the community level. Since malaria transmission has been reduced in the provinces surveyed, subpatent infections will remain undetected using microscopy as the operational standard of malaria diagnosis. It is important to identify communities experiencing small pockets of transmission using a more sensitive molecular tool comparable to PCR but one suitable for a “test &

treat" approach in a field setting. This may be useful in controlling and eliminating malaria even in endemic areas with poor geographic access in the Philippines.

From the output of this study, we developed a research proposal on "Application of novel nucleic acid surveillance to malaria elimination in South Cotabato Province, Mindanao, The Philippines" in 2013. The Malaria Eradication Scientific Alliance (MESA) in Barcelona, Spain funded this study and this is on going as collaboration between the London School of Hygiene and Tropical Medicine, the Nanyang Technological University in Singapore and the University of the Philippines Los Baños.

Screening for ACT and other drug resistance markers. This study only screened key genes that may influence *P. falciparum* and *P. vivax* sensitivities to ACT and chloroquine respectively. It will be useful to screen for both proposed and established molecular markers of artemisinin and lumefantrine resistance in *P. falciparum* isolates to protect the usefulness of ACT in this region. It is also important to further explore *pvmdr1* and other genes in assessing *P. vivax* resistance to chloroquine, which remains the first-line treatment option for vivax malaria in the Philippines.

Exploring diversity of *P. falciparum* and *P. vivax* in the Philippines. Polymorphisms in the *pfcrt* gene of *P. falciparum* isolates from Mindanao suggest that this population was likely different from other islands in the Philippines (Chen et al., 2003, Chen et al., 2005, Hatabu et al., 2009) but there is very limited information in local *P. falciparum* diversity. On the other hand, genetic diversity of *P. vivax* has not been previously studied at all in the Philippines. Using microsatellite markers, the population genetic structure of these two dominant circulating *Plasmodium* species in Mindanao should be determined and compared to other endemic provinces in the Philippines.

Determining the threat of *P. vivax* and other non-falciparum species to malaria control and elimination.

This study has shown that *P. vivax* persists in an area such as South Cotabato Province despite successful control measures against *P. falciparum* malaria and yet the extent of

threat of *P. vivax* to malaria elimination in the Philippines has not been studied. In addition, there is still evidence of *P. malariae* infection in Mindanao even if this species causes less than 1% of malaria cases in the country. While this study has not found human infections with *P. ovale* spp. and *P. knowlesi* in the surveyed population, it is not certain that these species are absent in Mindanao since these have been previously reported in the Province of Palawan and on the islands of Luzon (Alves et al., 1968, Luchavez et al., 2008). The simian host of *P. knowlesi* is present in all provinces surveyed and the Tawi-Tawi Province is near Sabah, Malaysia where *P. knowlesi* is most prevalent (Singh et al., 2004). There were anecdotal reports on movement of people between Tawi-Tawi islands and Sabah, which may influence transmission of *P. knowlesi*. These non-falciparum species may have important implications to malaria control and elimination in Mindanao and thus, the extent of their public health threat needs to be understood.

Appendices

Appendix 1. LSHTM Ethics Committee Approval

LONDON SCHOOL OF HYGIENE
& TROPICAL MEDICINE

ETHICS COMMITTEE

APPROVAL FORM
Application number: **5712**

Name of Principal Investigator	**Mary Grace Dacuma**
Department	**Infectious and Tropical Diseases**
Head of Department	**Professor Simon Croft**

Title: **Epidemiology of Plasmodium falciparum, P. vivax, and zoonotic P. knowlesi in Southern Mindanao, The Philippines: Geographic distribution, mutations in genes that confer resistance to drugs, and factors that influence emergence and transmission in the population**

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.

Appendix 2. National Ethics Committee of the Philippines Approval

NATIONAL ETHICS COMMITTEE
c/o Philippine Council for Health Research and Development
DOST Main Bldg. DOST Compound
Bicutan, Taguig City

3 October 2010

PROF. JUDELINE C. DIMALIBOT
Principal Investigator

PROF. MARY GRACE B. DACUMA
Co-Investigator

MR. GEORGE R. UGADDAN
Co- Investigator

Thru: DR. JAIME MONTOYA
Executive Director
Philippine Council for Health Research and Development

Dear Prof. Dimalibot, Prof. Dacuma and Mr. Ugaddan:

RE: Research Proposal, "Epidemiology of *Plasmodium falciparum*, *P. Vivax* and zoonotic *P. Knowlesi* in Southern Mindanao, The Philippines: Geographic dsitributions, mutations in genes that confer drug resistance, and factors that affect emergence and transmission in the population"

This is to acknowledge receipt of your response (last October 1) to the comments of the NEC based on a preliminary review of the proposal last May 2010.

The endorsements of Dr. Rachel Hallett (project supervisor), the Ethics Committee of the London School of Hygiene and Tropical Medicine Ethics Committee and the Independent Ethics Committee chaired by Dr. Angelica D. Francisco of De La Salle Health Sciences Institute are duly noted. The ethical clearance given by the latter committee has rendered further comments from the NEC unnecessary.

With warm regards.

Yours truly,

Marita V.T-Reyes

MARITA V. T. REYES
Committee Chair

Appendix 3. Participant Consent Form (English version)

Consent Form for Participation in the Research

Investigators' name and contact details:

Prof. Judeline C. Dimalibot
Animal Biology Division, Institute of Biological Sciences
University of the Philippines Los Baños
4031 Laguna, Philippines
Tel: 09209048226

Email: judimalibot@yahoo.com

Mary Grace B. Dacuma
Animal Biology Division, IBS
University of the Philippines Los Baños
4031 Laguna, Philippines
Tel: 09209280651

Email: Mary.Grace.Dacuma@lshtm.ac.uk

To be completed by participant

Please tick (√) as appropriate:

____1. I have read the information concerning this study (or heard it from the researchers, in case I can't read) and I understand what will be required of me if I take part in it.

____2. The researchers have answered my questions concerning this study.

____3. I understand that at any time I may withdraw from this study without giving a reason.

____4. I agree to take part in this study. (In the case of minors aged 12 months to less than 18 years of age: I am allowing my son/daughter/ward to take part in the study).

For the succeeding statements, please tick (√) any of the given options in each number:

5. In case that I am found positive for malaria through RDT, I give my permission to notify the Malaria Control Group of the province of my condition,

____a. And release my detailed information recorded in the Malaria Case Surveillance Report

____b. But will not permit the release of the detailed information recorded in the Malaria Case Surveillance Report

6. In case that I am found positive for malaria parasite with drug resistance or I am found positive for *P. knowlesi* in the laboratory, I give my permission to notify the Provincial Health Office (PHO) of my condition so they can find means to treat me accordingly, provided

____a. The PHO would notify me discreetly to protect my privacy and identity.

____b. The PHO would not publish my name or my exact household location.

Appendix 3. Consent Form (English version) continued.

I acknowledge receipt of information about the study and I am willing to participate in it:

Name of Participant……

Signature of Participant……

Date……..

Name of Witness……..

Signature of Witness……

Date……..

For participants who are less than 18 years of age:

I acknowledge receipt of information about the study and I am allowing my child or ward to participate in the study.

Name of Parent/Guardian……

Signature of Parent/Guardian……

Date……..

Name of Witness……..

Signature of Witness……

Date……

Appendix 4. Cross-Sectional Survey Questionnaire Form

QUESTIONNAIRE SURVEY

"Epidemiology of *Plasmodium falciparum*, *P. vivax*, and zoonotic *P. knowlesi* in Southern Mindanao, The Philippines: Geographical distribution, mutations in genes that confer resistance, and factors that affect emergence and transmission in the population"

Barangay: _____ Municipality: _____
Province: _____
Participant Number: |__|__|__|__|

- Use blue/black ink only
- Strike out wrong entries. Put initials and date on corrections.

INCLUSION CRITERIA	YES	NO
1. Aged 12 months and above	|__|	Stop*
2. Resident of the municipality for the last 6 months	|__|	Stop*
3. Has signed prior informed consent form (guardians/parents signature for the case of minors 12 months and above)	|__|	Stop*
EXCLUSION CRITERIA	**YES**	**NO**
With chronic disease not related to malaria and taking medication	Stop*	

* Do not include in the study.

A. DEMOGRAPHIC INFORMATION

1. Age *edad*	|__|__| years |__|__| months
2. Sex *kasarian*	|__| Male |__| Female
3. Weight *timbang*	|__|__|.|__|__| kg
4. Height *taas*	|__|__|.|__|__|m
5. Type of dwelling *uri ng tirahan*	Cement |__| Wood |__| Bamboo |__| Other(s) specify:
6. Sleeps under a bed net *Natutulog sa loob ng kulambo*	|__| Yes |__| No If yes: |__| ITN |__| non-ITN

B. SOCIO-CULTURAL INFORMATION

7. Ethnic Group *tribo* (specify)	
8. Occupation *hanapbuhay*	
9. Lives near the forest *nakatira malapit sa gubat*	|__| Yes |__| No
10. Frequency of going to or near the forest *dalas ng pagpunta sa gubat o malapit sa gubat*	|__| Always |__| Sometimes |__| Never
11. Type of dwelling *uri ng tirahan*	Cement |__| Wood |__| Bamboo |__| Other(s) specify:
12. Presence of monkeys near the house *Nakitang mga unggoy malapit sa tirahan*	|__| Yes |__| Not known |__| No

Appendix 5. Malaria Case Report (adapted from CDC, 2008)[14]

NOTE: The data recorded here will only be used for the purpose of this research and will not be released to the attending physician or interested third party without written consent of the patient.

Village : _____ Municipality: _____
Province: _____
Participant Number: |__|__|__|__|

Patient Name (last, first, middle initial): Date of symptom onset of this attack (dd/mm/yyyy): ____/____/_____	Age: |__|__| years |__|__| months Sex: |__| Male |__| Female Is the patient pregnant? |__| Yes |__| No
Travel history prior to illness? |__| Yes |__| No If yes, specify (municipality, province, country):	Patient referred to Provincial Malaria Control Group:|__| Yes |__| No Date: _____/_____/_____ (dd/mm/yyyy) Patient referred for hospital admission: |__| Yes |__| No Hospital:
Positive laboratory result (tick all that apply): |__| smear |__| RDT |__| PCR |__| No test done Species diagnosed (check all that apply): |__| *P. falciparum* |__| *P. malariae* |__| *P. ovale* spp. |__| *P. vivax* |__| *P. knowlesi* |__| Other species (specify)_____ Specimen sent to a reference laboratory? |__| Yes |__| No |__| Not known If yes, specify:	History of malaria in the last 12 months (prior to this report): |__| Yes |__| No |__| Not known If yes, species (check all that apply) |__| Falciparum |__| Vivax |__| Ovale |__| Malariae |__| Not determined |__| Other species (specify):_____ Complication from previous malaria: _____ |__| None Was previous illness severe? |__| Yes |__| No Recovered from previous illness? |__| Yes |__| No
Drugs taken (tick all that apply) |__| chloroquine (Aralen®) |__| sulfadoxine-pyrimethamine (Fansidar®) |__| artemether-lumefantrine (Coartem®) Other(s) specify: _____	
Were all the drugs taken as prescribed? |__| Yes |__| No, missed some doses If doses were missed, what were the reasons? |__| Forgot |__| Signs are gone, I feel well |__| No more supply of medicines |__| Side effect(s) specify_____ Other(s) specify	

[14] Adapted from the CENTER FOR DISEASE CONTROL 2008. Malaria Case Surveillance Report. Atlanta, Georgia: Center for Disease Control., with modifications.

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