Serological markers to measure recent changes in malaria transmission

A promising tool towards malaria elimination.

Karen Kerkhof

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Abbreviations

95% CI	95% Confidence interval
Abs	Antibodies
ACT	Artemisinin based combination therapy
Ags	Antigens
AMA1	Apical membrane antigen
ANC	Antenatal clinics
API	Annual parasite incidence
BSA	Bovine serum albumin
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
CNM	Cambodian government's National Centre for Parasitology, Entomology and Malaria Control
CSP	Circumsporozoite protein
DBP	Duffy binding protein
DDT	Dichlorodiphenyltrichloroethane
DGD	Belgian government
DNA	Deoxyribonucleic Acid
DRC	Democratic Republic of Congo
EBP	Erythrocyte binding protein
EIR	Entomological inoculation rate
ELISA	Enzyme Linked ImmunoSorbent Assay
FOI	Force of infection
FSAT	Focused screening and treatment
GEE	Generalized estimating equation
GLM	Generalized linear mixed models
GLURP	Glutamine rich protein
GMS	Greater Mekong Subregion
HAs	Health areas
HIV	Human Immunodeficiency virus
HZs	Health zones
IFAT	Immunofluorescence assay technique
IFN-γ	Interferon gamma
Ig	Immunoglobulins
IL-10	Interleukin-10
IL-12	Interleukin-12
IP	Institut Pasteur

IPC	Institut Pasteur du Cambodge
IPT	Intermittent preventive treatments
IRR	Incidence Rate Ratio
IRS	Indoor Residual Spraying
ITM	Institute of Tropical Medicine Antwerp
ITNs	Insecticide treated nets
LAMP	Loop-mediated isothermal amplification
LLINs	Long lasting insecticidal nets
Ln	Natural logarithm
LRT	Likelihood ratio test
LSA	Liver-stage antigens
malERA	Malaria Eradication Research Agenda
MBCs	Memory B-cells
MDA	Mass drugs administration
MDG	Millenium Development Goals
MDR	Multi drug treatment
MFI	Median Fluorescent Intensity
MIS	Malaria information system
MoH	Ministry of Health
MPA	Multiplex assay
MRDD	Malaria Rapid Diagnostic Device
MSP	Merozoite surface protein
NKs	Natural killer cells
OR	Odd's ratio
PCD	Passive case detection
PCR	Polymerase chain reaction
Pf13	*Plasmodium falciparum* 13
PHF	Public Health facilities
PP	Percentage positivity
PR	Parasite Rate
RBCs	Red blood cells
RBM	Roll back malaria
RCT	Pre-randomized controlled trial
RDTs	Rapid diagnostic tests
Ro	Basic reproduction rate
RSD	Relative standard deviation
RTS, S	Mosquirix^{TM} (malaria vaccine)
SALSA	Sporozoite- and liver stage antigen

SCR	Seroconversion rate
SD	Standard deviation
SMC	Seasonal malaria chemoprevention
SP	Sulfadoxine-pyrimethamine
SR11.1	Subrregion antigen 11.1
SRR	Seroreversion rate
STARP	Sporozoite threonine- and asparagine rich protein
TB	Tuberculosis
USA	United States of America
VFCV	V-fold cross-validation
VIF	Variance Inflations Factors
VMW	Village Malaria workers
WHA	World Health Assembly
WHO	World Health Organisation

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INTRODUCTION

Rationale

Areas with a low prevalence and incidence of malaria, such as Cambodia, pose considerable challenges for epidemiological surveillance and evaluation. Detection of parasitological indicators by microscopy and to lesser extent by PCR lack sensitivity because of the presence of asymptomatic cases with low parasitaemia [1, 2], representing a formidable logistical challenge due to very big sample sizes required. Parasite-prevalence (measured by microscopy, RDTs or PCR) provides a snapshot of the exposure to malaria at a certain moment. Seroprevalence on the other hand provides a picture of the malaria transmission over a prolonged period. This can be relatively short or long, depending on the serological marker used [3]). Seroprevalence is based on the detection of antibodies against antigens of *Plasmodium* parasites. This offers the advantage that anti-*Plasmodium* antibodies can persist for months after infection. Therefore these antibodies have been suggested as indicators of malaria transmission [4, 5], and are believed to be more sensitive in detection of past, recent and present malaria exposure [1]. A multiplex immunoassay detects a panel of antibodies. Combining long- and short-lived markers in one test will provide knowledge about pas and recent changes in malaria transmission [3, 6].

The current PhD thesis is part of the large scale project "MalaResT" entitled 'Repellents as added control measures to long lasting insecticidal nets to target residual transmission in Southeast Asia: a step forward to malaria elimination' and of the research project "MalaSpot entitled 'Eco-epidemiology of asymptomatic malaria hotspots, a perquisite for malaria elimination?'. Both projects are collaborative projects between the Unit of Medical Entomology at ITM, the 'Cambodian National Malaria Control Program' (CNM) and 'Institut Pasteur du Cambodge (IPC). The collaboration between these three institutes is formalized on the long-term starting from 2014 in a framework agreement funded by the Belgian government (DGD), in which research to study malaria heterogeneity and to evaluate innovative vector control tools and strategies are the main scientific interests.

The MalaResT project aimed to raise evidence on the effectiveness of the mass use of topical repellents in addition to long lasting insecticidal nets (LLINs) to reduce malaria infections [7, 8]. In this intervention trial, 113 of the most endemic villages in Ratanakiri were randomly assigned to a control arm, in which every household received one LLIN per person, or to an intervention arm, where in addition to LLINs one bottle of topical repellents were distributed biweekly per person. The secondary

outcome of the MalaResT project is to test for difference in malaria exposure between the control and the intervention arm based on serological markers.

The MalaSpot project aims to identify and characterize malaria hotspots that are stable in time at micro-geographical level through a spatiotemporal statistical approach. Within this project, the serological data obtained in the MalaRest Project will be used as village based measure of exposure to Falciparum, Vivax and Malariae malaria in the spatiotemporal analysis. This information will be combined with the information on landscape/soil occupation, human related factors (socio-economic status, health behaviour & genetics), parasite related factors, vector related factors, and climate data, as well as the epidemiological data on the prevalence and incidence of Falciparum and Vivax malaria as measured by PCR and passive (Rapid Diagnostic Tests) case detection. With this combination unparalleled geostatistical analysis of malaria hotspots for Vivax and Falciparum malaria can be performed, which was never possible (especially for Vivax malaria).

Objectives

The **central question** of this PhD thesis is whether a multiplex immunoassay has additional value in the monitoring of (ongoing) malaria transmission, and thereby detecting changes in malaria transmission intensity due to seasonality or interventions in a hypo-endemic transmission setting.

Specific objective 1: To implement a multiplex immunoassay and document its value as compared to other serological tests.

I. Literature review in which the multiplex serology (Luminex) is compared to the immunofluorescence assay (IFAT) technique.
II. Implementation of the multiplex immunoassay for detection of antibodies against antigens of different malaria parasites.
III. Documenting the half-life of the used serological markers by analyzing sequential and cohort samples from individuals in Ratanakiri province, Cambodia.

Specific objective 2: To validate multiple serological markers for estimating malaria transmission.

IV. Changes in malaria transmission due an intervention trial.
V. Geographical clustering of malaria transmission.

Project outlines

The current PhD thesis is built in a variety of steps.

Chapter 1 describes the rationale and the aims and hypotheses of this thesis

Chapter 2 presents information about the malaria disease, its global burden and the past and current control and elimination efforts. Subsequently, information about the different malaria detection tools is disclosed, with the emphasis on the use of serological markers with a multiplex immunoassay. Thereby, this chapter makes clear that serology can only be used in an epidemiological context and not as a detection tool. And finally, a detailed overview of the study area, Cambodia, is presented.

Chapter 3 discusses about the differences between the classical serological tools (IFAT) with the modern high throughput techniques (Luminex). The IFAT is a very cumbersome tool, but takes into account the whole antigenic battery of the parasite, while in the Luminex only a selection of antigens are taken into consideration

- **Kerkhof K,** Coosemans M. **Serological tools for malaria: what are the potentials?** [In progress]

Chapter 4 describes the implementation of a highly performant, standardized and reproducible serological test for detecting multiple serological markers for malaria infection by different parasites. The focus mainly lies on the assessment of the main features of the assay (e.g. repeatability, bead-interactions, selection of additional peptides and recombinant proteins) and its application to samples collected in the MalaResT project in Cambodia [9].

- **Kerkhof K,** Canier L, Kim S, Heng S, Sochantha T, Sovannaroth S, et al. **Implementation and application of a multiplex assay to detect malaria-specific antibodies: A promising tool for assessing malaria transmission in Southeast Asian pre-elimination areas.** *Malar J* 2015, **14**:338

Chapter 5 characterizes the use of the serological assay to improve the understanding of the effectiveness of additional use of topical repellent on malaria incidence in a community based trial. Therefore a generalized linear model was carried out on the serological data to compare the control and intervention arm from the large-scale project (MalaResT) where this thesis is part of. The analysis was based on a random selection of samples from 2 surveys from the MalaResT project conducted in 2012 and 2013. As a result no difference could be observed in the seroprevalence outcome between the control and intervention arms. These finding were in line with the

preliminary results from the PCR prevalence and Passive Case Detection. As such serology confirms the findings of the primary outcome of the MalaResT project. Results were processed in the end publication from the MalaResT project and the manuscript has been submitted to the Lancet [10].

- Sluydts V, Durnez L, Heng S, Gryseels C, Canier L, Saorin K, Van Roey K, **Kerkhof K**, et al. E**fficacy of topical mosquito repellent (Picaridin) plus long-lasting insecticidal nets versus long-lasting insecticidal nets alone for control of malaria: a cluster randomized controlled trial.** *Lancet Infect dis* 2016, **16**:1169.

Chapter 6 documents the half-life and defines the sensitivity of serological markers to pick up recent (sporadic and seasonal) changes in malaria transmission over time. Hereby, the focus lays on determining a clear indication of which marker should be used to measure short-term (seasonal) changes in force of infection in low endemic areas. To reach this goal three different approaches are used; (1) comparing Ab-responses based on the differences in ln(MFI) between PCR positive and PCR negative individuals, (2) assessing the half-life of the Abs against the *Plasmodium* Ags, and (3) observing the Ab-responses in relation to high-, medium-, and low levels of *Plasmodium* exposure [11].

- **Kerkhof K,** Sluydts V, Willen L, Kim S, Canier L, Heng s, et al. **Serological markers to measure recent changes in malaria at population level in Cambodia.** *Malar J* 2016, **DOI**:10.1186/s12936-016-1576-z.

Chapter 7 evaluates the potential of this serological assay identifying the remaining geographical clusters of malaria transmission. In malaria endemic countries parasites tend to linger in small areas or populations (hotspots/hotpops). It is assumed that there will be no persistence of malaria transmission when these recurrent stable sources are eliminated [12]. Therefore, identifying and targeting these geographical clusters is a very effective malaria control strategy. So far, hotspot identification has mainly been carried out based on microscopy and PCR parasite prevalence. However, due to the low sensitivity of these methods in estimating malaria transmission in hypo-endemic areas, these methods can be supplemented with the serological data for the identification of geographical clusters [13].

- **Kerkhof K,** Sluydts V, Heng S, Kim S, Pareyn M, Willen L, et al. **Geographical patterns to identify past and present asymptomatic hotspots of malaria transmission in Ratanakiri, Cambodia.** *Malar J* 2016, **15**:510

Chapter 8 gives an extensive overview about the main findings of this thesis research. It focusses on the implementation of the serological assay, the added value of this assay, the evolution of modelling approaches, the use of serology at geographical scale (spatial clustering), and finally the added value of all these approaches towards malaria control in Cambodia and other areas.

Chapter 9 comprises the future perspectives that have occurred in this thesis

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BACKGROUND ON MALARIA (SERO) PREVALENCE AND TESTING

1. Malaria in general

Malaria, a parasitic disease present in vertebrates, is the most common vector-borne disease in the animal world [1], caused by a protozoan parasite of the genus *Plasmodium*. Malaria disease occurrence varies widely and accounts together with Human Immunodeficiency virus (HIV) and Tuberculosis (TB) for a vast majority of death from infectious diseases [2]. As of 2015, spread over 97 countries 3.2 billion people are at risk [3], of which 1.2 billion people at high risk, mainly in the African (53%) and Southeast Asian (29%) regions [3]. Moreover, worldwide around 198 million cases were reported with an estimated 584,000 deaths, of which 78% of the deaths occurred in children under the age of five [3]. When looking at malaria transmission a distinction can be made between stable and unstable malaria. In areas with a constant, intense, year-round infection, the term stable malaria is used. In these regions the prevalence of malaria infection does not fluctuate according to seasons, despite fluctuations in transmission intensity and morbidity [1]. Clinical malaria affects mainly young children (under the age of five) and pregnant women, and less other adults due to an acquired protective immunity [1]. On the contrary, in regions with unstable malaria, transmission intensity tends to be low, erratic or focal and the prevalence of malaria infection varies significantly from year to year [1, 4]. In these regions epidemics can occur when changes in environmental, economic or social conditions emerge together with a breakdown in malaria control and prevention [4]. These epidemics affect all ages, due to a low or almost absent acquired immunity [4].

The disease is characterized by recurrent attacks of high fever including chills, sweating, headaches, severe malaise, joint and muscle pains [1, 5]. In some cases, this disease can lead to life threatening complications, such as cerebral malaria or severe malaria anemia [1, 6].

Human malaria can originate from one of four *Plasmodium* species: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, and *Plasmodium malariae*, although a fifth species *Plasmodium knowlesi* known to cause malaria amongst monkeys (zoonosis) can also infect humans [4, 7].

Among these *Plasmodium* species, *P. falciparum* causes the most severe form of malaria that can be fatal and predominates especially in Africa [4, 8]. *P. vivax* causes significant symptoms that are rarely fatal compared to *P. falciparum*. It has a wider geographical distribution (the American Region, the Western Pacific region and in some parts of Southeast Asia) [3, 4, 9], but is less frequently found in Africa due to the absence of the duffy antigen required for invasion of the red blood cells [10]. *P. ovale*

is mainly seen in the sub-Saharan Africa and in the Western Pacific region and hardly anywhere else [11]. Both *P. vivax* and *P. ovale* are noted to produce dormant liver stages (hypnozoites (Figure 1-1)) which can be reactivated weeks to years after a primary infection to cause relapses [10]. On the other hand a *P. malariae* infection also often leads to these latent malaria infections although it is not associated with the production of hypnozoites [1, 10].

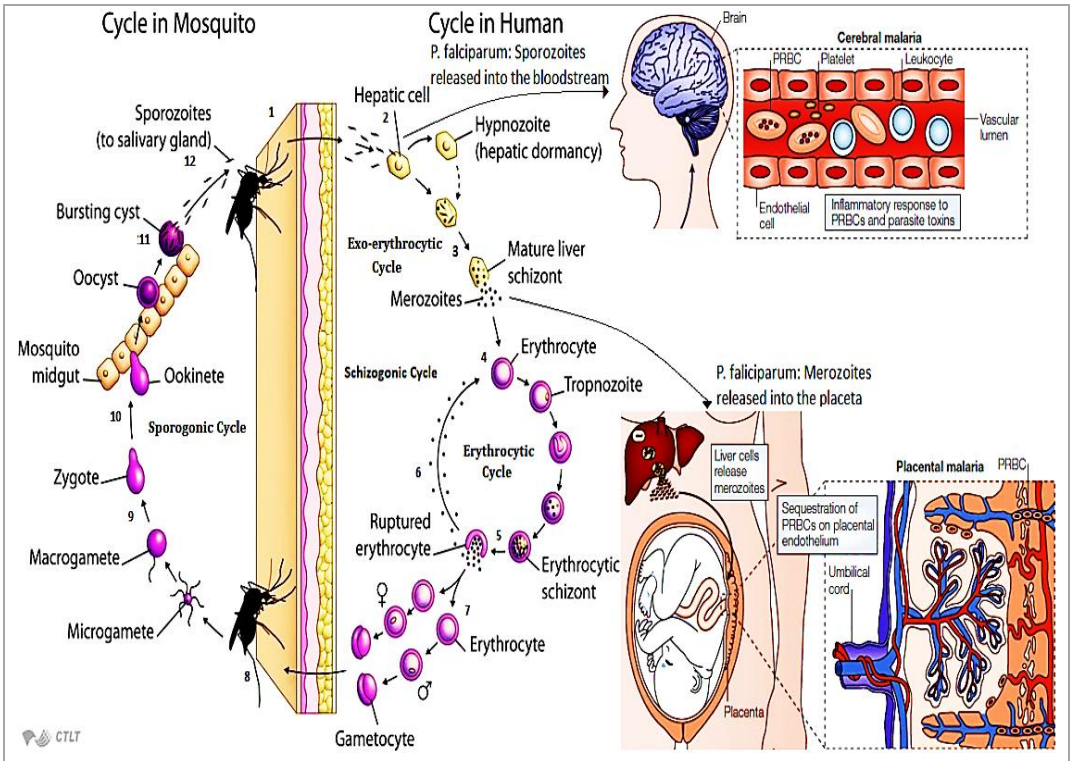

Figure 1-1: *Plasmodium* parasitic life cycle [12]**, highlighting *P. falciparum*** [13]**; 1. Female *Anopheles* mosquito injects sporozoites into the skin (schizogonic cycle); 2. Sporozoites migrate to the liver and infect hepatocytes** [4, 14]**, where they multiply and convert into schizonts; 3. Within 8 till 10 days the schizonts release the merozoites [10,000 – 30,000] into the blood circulation** [1]**; 4. Merozoites invade the erythrocytes (erythrocytic cycle) and convert into ring-stage trophozoites** [1]**. These become erythrocytic schizonts that produce approximately 16-32 merozoites** [4]**; 5. Merozoites are released after 48 hours (*P. falciparum*, *P. vivax* and *P. ovale*) or 72 hours (*P. malariae*); 6. From this moment on the human host will experience an increase in body temperature, resulting in the typical recurrent fevers and thereby perpetuating and promoting the schizogonic cycle** [4, 14, 15]**. Symptoms will appear around 12-14 days after the infection and when the parasitic densities have reached 50 µL blood** [1]**; 7. Some merozoites convert into male and female gametocytes; 8. When a mosquito bites the human host again, it ingests the gametocytes (sporogonic cycle)** [1, 4]**; 9. Gametocytes enter the midgut, in which male gametocytes generate microgamets by exflagellation that further fertilise macrogametes that produce zygotes; 10. These transform into a slow motile ookinete, which infiltrates the midgut and mature into oocysts; 11. The oocytes produce approximately 8,000 sporozoites that burst out of the oocysts and; 12. migrate to the salivary glands to continue the *Plasmodium* life cycle by infecting the next human host** [4, 14]**. A female mosquito remains infectious for her entire life** [14]**. (Adapted from: Johns Hopkins University** [12] **and Schofield *et al.*** [13]**).**

Malaria parasites are transmitted through the bite of an infectious female mosquito [4]. Globally 3,000 mosquito species have been described. From these, 444 formally named species and 40 provisionally designated extant species were recognized as distinct morphological and/or genetic species of *Anopheles* [4, 16]. Approximately 60 are considered to act as good vector of human malaria parasites [17].

Malaria vectors generally bite between sunset and sunrise, but exceptions may occur [18]. The *Plasmodium* life cycle shown in figure 1-1 is a complex process in its human host as well as in its vector. The life cycle starts when sporozoites are injected into the human host (asexual phase) and trigger the schizogonic cycle. This process occurs directly when it concerns *P. falciparum* and *P. malariae* sporozoites. However, a delayed liver schizogonic cycle can occur with *P. vivax* or *P. ovale*, due to the hypnozoite stage. After the asexual phase the sexual stage (sporogonic cycle) is completed in the mosquito [4, 7]. The basic elements within the life cycle are the same for all *Plasmodium* species.

2. From malaria control to malaria elimination

2.1 Malaria control strategies

Malaria control receives high priority on the international community agenda as one of the United Nation's Millenium Development Goals (MDG). Malaria control is central to MDG 6. In 2005, the World Health Assembly (WHA) urged for a 75% reduction in the incidence of malaria and other diseases by 2015 [19]. Additionally, it is also of great importance to MDG 4 that focuses on a two-third reduction in the mortality rate among children under the age of five [3]. Finally, the vision of the World Health organization (WHO) is a world free of malaria [20]. In practice, malaria control programs are based on three main components: vector control, chemoprevention and case management (Figure 1-2) [3].

2.1.1 Vector control

Vector control is one of the key elements in the fight against malaria, with the aim to reduce the burden of malaria by cessation of parasite transmission between the human host and the mosquito. The most effective part that contributed to a global reduction of malaria was scaling up existing control measures, such as the roll out of insecticide treated nets (ITNs) and indoor residual spraying (IRS) [3, 18].

ITNs include both conventional nets treated with an insecticide every few months and the newer long lasting insecticidal nets (LLINs). LLINs are factory-treated nets. The insecticide is incorporated or coated onto the fiber and is expected to retain bioactive

for at least three years [21]. These nets provide a good personal protection against malaria, but especially leads to a mass effect (by affecting mosquito survival) when used at large scale. This means that people not sleeping under a net are also protected from malaria in communities with high bed net coverage [22]. Recent considerable efforts have been made by most of the malaria-endemic countries to promote universal access to ITNs for those who need it [3]. In order to achieve sustainable coverage, distribution should employ a combination of both catch up and keep up programme strategy. The catch up strategy rapidly scales up coverage through periodic mass distribution of ITNs, while the keep up strategy maintains coverage levels through routine delivery channels such as antenatal clinics (ANC) [23, 24].

IRS for vector control is an operational intervention effective in controlling malaria transmission and to reduce the burden of malaria disease. According to the WHO, this is only possible when >80% of the houses in the target areas are treated. IRS involves application of insecticides inside houses to kill the mosquitoes that tend to rest after taking a blood meal [3]. During the "Global Malaria Eradication Program", IRS with dichlorodiphenyltrichloroethane (DDT) was the primary malaria control method used. Eventually the use of DDT lead to a concern over the environmental impact and newer insecticides were introduced [8]. As a result of the high cost, the failure of the campaign and the environmental concerns, IRS became negative publicity and was disbanded from most countries for agriculture purposes. However, some South-African countries continued with using DDT, carbamates or pyrethroids for IRS and significantly improved malaria control. This revived the interest in using IRS for vector control again [8].

2.1.2 Chemoprevention

Preventive chemotherapy uses complete treatment courses of antimalarial medicines as prophylaxis in populations at risk of malaria. It prevents malaria disease inside the human host by suppressing the blood stage infection, which eventually reduce malaria-related morbidity and mortality [3]. For the most susceptible population (young children and pregnant woman) protection could be realized with intermittent preventive treatments (IPT). Therefore three different types of chemoprevention are known, namely IPT in pregnancy (i.e. administration of sulfadoxine-pyrimethamine (SP)), IPT in infants and seasonal malaria chemoprevention (SMC) for children aged 3-59 months (i.e. amodiaquine in combination with SP) [1, 3]. IPT in pregnancy can reduce severe maternal anaemia, low birth weight and perinatal mortality, when performed during the second and third trimester of pregnancy [3]. For infants IPT is routinely performed inside the immunization clinics. It reduces hospitalizations for

malaria infected children, as it provides protection during their first year of life [3]. In areas with a high seasonal malaria transmission SMC could avert the high mortality rates during the high malaria season when maintaining therapeutic antimalarial drug concentrations in the blood [3].

2.1.3 Prompt diagnosis and rapid treatment (case management)

Case management is a keystone of malaria control strategies and is used to detect, diagnose, treat and cure malaria infections to reduce the likelihood of progression to severe disease and death from malaria [3]. Proper diagnoses and treatment with appropriate antimalarial drugs can (i) prevent development of simple malaria into severe and potentially fatal cases and (ii) prevent malaria-related anaemia, due to a chronic infection [25, 26]. As a first step blood samples are checked by microscopy or rapid diagnostic tests (RDTs) as not all fevers indicate a malaria infection. If malaria is identified people will be treated with antimalarial drugs within 24 hours [2, 3, 26]. The most common and recommended treatment for *P.falciparum* is the use of artemisinin-based combination therapy (ACT). In case of a *P.vivax* infection ACTs or chloroquine is sometimes used in combination with primaquine [3]. This combination is needed to prevent relapses caused by de latent hypnozoites in a *P.vivax* infection.

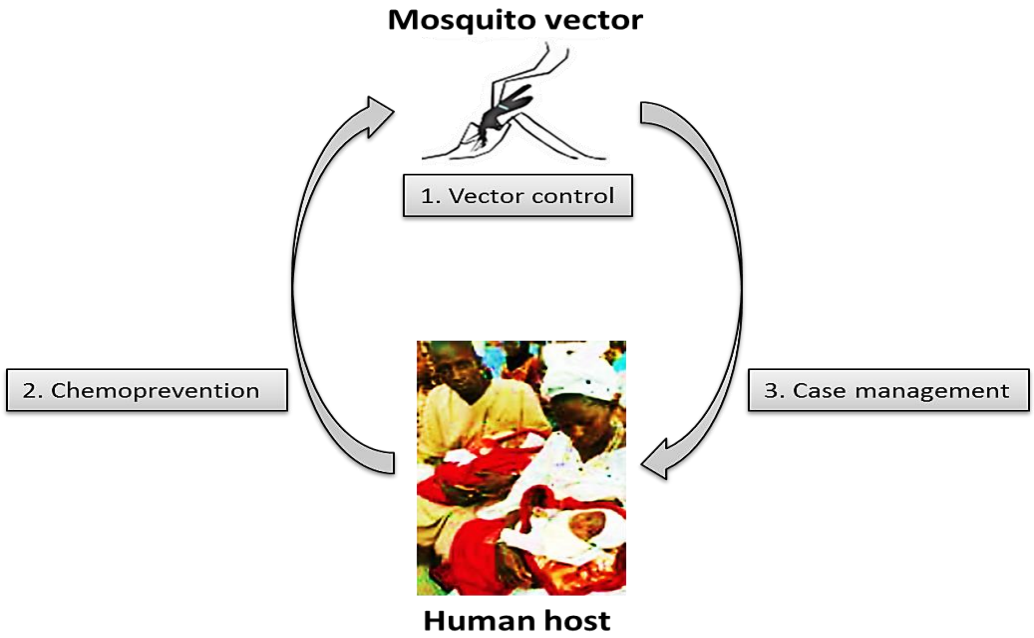

Figure 1-2: Main strategies to prevent and treat malaria [19]**. 1. Vector control to prevent a mosquito of passing on an infection; 2. Chemoprevention is used to prevent malaria infections in the human host; 3. Case management involves diagnosis and treatment to reduce the progression of severe malaria and mortality rates.**
(Adapted from: World Health Organization [19]**).**

2.2 The Global Malaria Eradication program and its history

Malaria control, pre-elimination, elimination, prevention of reintroduction and eventual eradication are very topical concepts [27] (see Figure 1-3). The notion of eradication dates back to the 18th century, when curative and prophylactic "quininisation" was the basis of an effective battle against malaria. At the end of the 19th century, England was malaria free. Nevertheless, malaria persisted in Italy, Corsica, Greece, The Balkans, The Netherlands, Germany, and the United States of America (USA) until 1945 and in the Soviet Union until 1959, despite the significant relapse due to World War I and II [4].

Another period in the eradication era started in 1955, when the WHO launched the "Global Malaria Eradication Program" [28]. This program focused mainly on using DDT for indoor residual spraying (IRS) and mass drug administration (MDA) [25]. MDA is not currently recommended but was part of many malaria elimination campaigns during the eradication era [28, 29]. MDA can be technically defined as an experimental administration of drugs to an entire population at the same time [29]. It proved to be successful in several countries [28, 29], as the Garki project in Northern Nigeria in 1969 where MDA with sulfalene-pyrimethamine was combined with IRS [30] and in China and Vanuatu [28]. This ambitious campaign resulted in the elimination of malaria in all developed countries and even different regions in Asia and Latin America [27]. However, it was abandoned in 1969 when appeared that it was not strong and consistent enough to achieve global eradication, due to administrative, financial and technical issues [25, 28]. The partial failure of this strategy was according to Alonso et al. (2011) [27] primarily due to (1) the poorly known heterogeneous nature of malaria and its vectors, (2) the lack of available knowledge and the use of insufficient and unsuitable tools to eliminate malaria, and (3) the inadequate investment in research and local applications of research findings [27].

In 1992, global recognition of malaria returned, with the advent of a new strategy for malaria control. This new strategy comprised four fundamental technical elements: (i) prompt diagnosis and rapid treatment; (ii) planning and implementing selective and sustainable preventive measures, including vector control; (iii) early detection, containment and prevention of epidemics; and (iv) strengthen local capacities in basic and applied research [31].

From 1993 till 2000 a global plan of action was carried out as guidance for the implementation of the global malaria control strategy. This strategy arose as a result

of the lack of involvement of the public and private sectors, as well as on communities and individuals at risk [31, 32].

Until this moment ITNs treated with long-acting insecticides as a personal protection was considered a promising tool to combat malaria [31]. However, according to the observed low re-treatment practices of ITNs, the WHO highly encourages the use of LLINs [3]. LLINs are designed to kill mosquitoes when they encounter people or houses [33], as insecticide is incorporated or coated onto the fibers of the nets [21]. Washing and variation in insecticide dosing is considered as a major breakthrough in malaria prevention [3, 34].

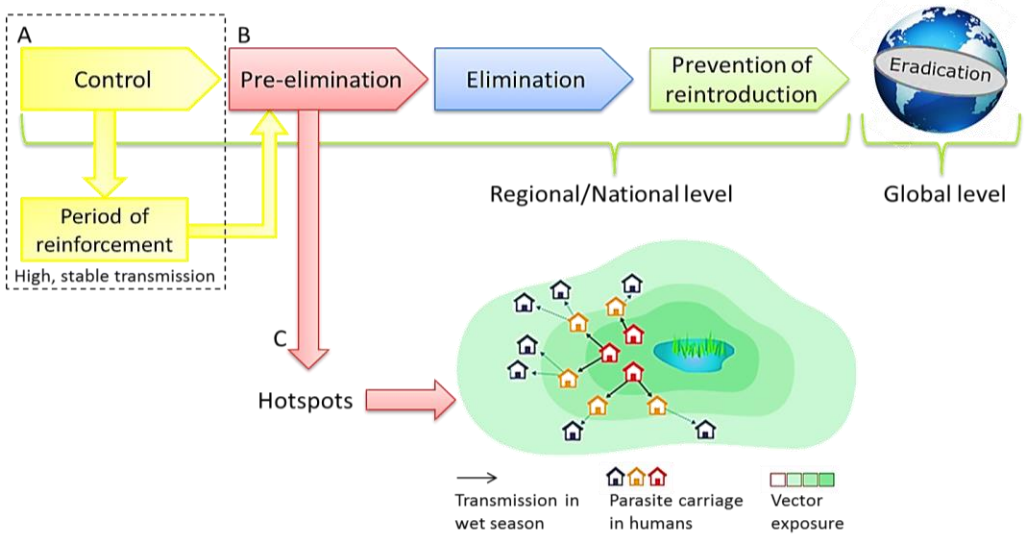

Figure 1-3: Epidemiological steps to obtain eradication [25, 27, 35]**. A: The subsequent milestones a country has to undergo to eventually achieve 'prevention of reintroduction'. B: Global eradication can be achieved when all countries eliminated malaria** [25, 27]**. C: A difficulty that a country will face is dealing with the remaining malaria hotspots, which form a reservoir of the parasite and subsequently fuel malaria transmission to wider areas. People living in hotspots are exposed to higher mosquito densities (dark green area). Individuals from households inside the hotspots (red houses) are more likely to be infected and become infectious, due to the persistent vectors. In these households it is more likely that mosquitoes acquire a malaria infection. Highest malaria transmission is seen during the rainy season, when the mosquito density increases and infectious mosquitoes drive the infection to wider areas outside the village (from red to yellow and eventually black houses)** [35]**.**
(Adapted from: (A) Alonso *et al.* [27]**; (B) Mendis *et al.*** [25] **and (C) Bousema *et al.*** [35]**).**

Definitions:
Control: reduction of disease incidence, prevalence, morbidity and mortality to a local acceptable level. To maintain this reduction constant intervention measures are needed [27]**.**
Elimination: reduction in incidence of locally transmitted malaria infection to zero in a well-defined geographical region. To prevent reintroduction of transmission ongoing intervention measures are needed [27]**.**
Eradication: lasting reduction in global malaria incidence to zero. Intervention measures are no longer needed [27]**.**

From 1998 the Roll Back Malaria (RBM) partnership with the subsequent support of the WHO was established aiming to a 75% decrease in malaria cases by 2015 [3]. Based on the RBM strategies, the Abuja Declaration followed in 2000. This was a declaration setup by 44 malaria-affected African countries targeting a 60% protection and treatment of the African at-risk population to obtain a decrease in malaria mortality by 2010 [36]. The fight of the RBM was reinforced by the discovery of Artemisinin-based recombination therapy (ACT), as the number of resistant parasites to anti-malarial drugs increased [36].

An important step in the fight against malaria was the Global Fund. This Fund was launched in 2002 to attract, manage and disburse financial resources to reduce the impact of HIV/AIDS, TB and malaria [8, 24]. In October 2007 the Bill & Melinda Gates Foundation, with the support of the WHO and RBM partnership, brought malaria eradication back on the agenda with the goal to achieve a zero death from malaria situation by 2015 [27]. Therefore, they grouped the countries aiming for elimination into four different phases; the control phase, pre-elimination phase, elimination phase and the phase of prevention of reintroduction (Figure 1-3) and took into account the regional discrepancies in malaria epidemiology [3]. Roughly 50% of the countries are on the right path to achieve elimination, due to large-scale and rapid scale-up of effective malaria control interventions [3].

In order to define the critical knowledge base, strategies and tools required to reduce malaria transmission, the 'Malaria Eradication Research Agenda (malERA) initiative' was introduced [27, 37]. The insight of the malERA initiative was based on a research and development agenda for regional elimination and eventual global eradication of malaria. It was built up by more than 250 scientists from 36 different countries. They communicated in the form of a series of articles in which they identified key knowledge gaps, strategies and transformational tools necessary to reduce malaria transmission to finally achieve eradication [27].

At present time, the fight against malaria still evolves towards the ultimate goal of a world free from malaria. Therefore the WHO with the support of the RBM partnership developed a Global technical strategy for malaria 2016-2030 to address remaining and emerging challenges. The visions of this new strategy towards 2030 include: (i) Reduce malaria mortality rates with 90% compared to 2015; (ii) Reduce malaria case incidence with 90% compared to 2015; (iii) Eliminate malaria in at least 35 countries in which malaria was transmitted in 2015; and (iv) Prevent re-establishment of malaria in all countries that are malaria free [19].

2.3 Knowledge gaps and challenges in the combat against malaria

The only known human disease that have ever disappeared through the high commitment of people, was the small-pox, which was eradicated by the efficient use of highly effective preventive tools (a vaccine) and the existence of the close correlation between the disease and infection [38]. Unfortunately, neither of these features exist for malaria, which leaves us with major knowledge gaps and challenges still to overcome in the elimination progress [38].

A first pillar is that a completely effective vaccine does not yet exist for malaria, although one malaria vaccine (RTS, S) became registered since July 2015 [39]. This is a recombinant vaccine that consists of the *P. falciparum* circumsporozoite protein which targets the pre-erythrocytic stage. It is developed to produce antibodies (Abs) capable of preventing the invasion of hepatocytes [40].

A second pillar is the vector control strategy for malaria prevention which also faces major challenges to prevent malaria transmission. Current vector control tools, as ITNs or LLINs only target indoor- and night biting mosquitoes, while IRS only affects the resting indoor mosquitoes [18]. As a result there is a 'gap' in protection just before bed time and for people who remain outdoors during the night [18, 38]. Residual malaria transmission is, therefore defined here as all forms of malaria transmission that persist when full global coverage with effective ITNs/LLINs and IRS interventions have been achieved. For that reason it is recommended to complement these vector control tools with other new innovative strategies [33] for controlling and/or interrupting malaria transmission if malaria elimination is the final aim. These new tools must be developed to target outdoor and early biting mosquitoes and should be available for all populations at risk [18]. The entomological inoculation rate (EIR) is currently the golden standard for monitoring malaria transmission intensity. It corresponds to the number of infectious mosquito bites per person per unit of time [41, 42]. It is important to keep in mind that it is difficult to measure the EIR in pre-elimination areas, where vector density and/or sporozoite rates are very low (EIR is often between 1-5 infective bites per year) [42–44]. Alternatively, the parasite prevalence by microscopy or PCR in a given population [43, 45, 46] or the force of infection (FOI) parameter, that corresponds to the number of infections acquired per person per year [41] are used as a proxy of malaria transmission.

A country has to face many obstacles to eventually reach malaria elimination. When a country is situated in the malaria pre-elimination phase, large varieties in transmission and incidence of malaria are seen between different areas [25, 27]. In this phase the

parasite reservoirs seems to linger in smaller areas (hotspots), causing a highly heterogeneous transmission of malaria [47]. In some regions, the reported malaria cases are demographically clustered according to social and behavioral features, which are called hot-populations (hotpops). These hotspots or hotpops are the source for transmission within small areas or populations [47]. In case of complete clearance of the *Plasmodium* parasites from a given area, it is essential to include massive screening campaigns, radical cure treatment for infected individuals or immediately treating an entire community at-risk. Therefore, improved and more sensitive diagnostic tools are needed, especially, those that can identify carriers of low parasite burden. These may signify great benefits for early treatment of parasite carriers, as well as for prevention of reintroduction and maintenance of zero transmission [38].

With this in mind, **a third pillar of malaria elimination relies on the surveillance system** [38]. Surveillance is the key component of malaria control, and becomes even more important in elimination programs as parasite reservoirs need to be identified [45, 48, 49]. So far it has been reliant on passive case detection, entomological estimates and parasite prevalence (microscope or PCR). However, detection of malaria infected persons by microscopy, RDTs and even PCR lacks sensitivity, when parasitaemia is low to detect sub microscopic reservoirs [50, 51], representing a formidable logistical challenge due to very big sample sizes required.

While parasite-prevalence (measured by microscopy, RDTs or PCR) provides a snapshot of the exposure to malaria at a certain moment, seroprevalence offers a picture of the malaria transmission over a prolonged period (can be relatively short or long, depending on the serological marker used [5]). Seroprevalence is based on the detection of Abs against antigens (Ags) of malaria parasites. These offer the advantage that anti-*Plasmodium* Abs can persist for months after infection. Therefore, these Abs have been suggested as indicators of malaria transmission [2, 52], and are believed to be more sensitive to determine past, recent and present malaria exposure [50].

For moving from malaria control to malaria elimination, the development and application of serological markers of malaria exposure in sero-surveillance tools provide valuable information [53]. Abs to malaria Ags are sensitive markers of malaria transmission on population level. They can be used to identify hotspots, estimate the force of infection (FOI), monitor changes over time (i.e. the impact of intervention trials), confirm malaria elimination, and monitor reappearance of malaria disease [45, 54]. For community-based surveillance sero-surveillance tools might be developed as simple point-to-care tests. To improve the implementation of sero-surveillance tools

for malaria-elimination, major knowledge gaps and challenges need to be tackled. Therefore, new research should include more information about potential Ags, the sensitivity and specificity of Ab-responses, the longevity (half-life) of these responses and defining Ags and Abs that distinguish between exposure to *P. falciparum* and *P. vivax*. Additionally, a better understanding of the influence of host factors (i.e. age, genetics and the simultaneous presence of different Ab-responses) in different populations is needed when aiming for malaria elimination [47].

3. Malaria parasite detection methods in humans

In case of clinical, epidemiological and research purposes an accurate diagnosis of malaria is crucial [2]. The mainly used methods to diagnose malaria are directly performed on blood samples to detect the living parasite, Ags or DNA [4]. These methods are: conventional microscopic diagnosis by staining thin and thick blood smears, Rapid Diagnostic Tests (RDTs) and molecular diagnostic methods (i.e. polymerase chain reaction - PCR) [2, 4].

3.1 Conventional microscopic diagnosis

The oldest and still routinely used method to estimate the parasitaemia is conventional microscopic diagnosis by staining thin and thick blood smears [2, 4]. Even though only a minimum of equipment is required (staining solution and microscope with immersion lens) this technique must be carried out with precision [4]. Therefore, an intensive training is required. This technique is based on two drops of blood taken from a patient's finger and put on a glass slide. A thick blood smear is just a drop of blood on a glass slide, while for a thin blood smear the blood is spread across the area of the slide. Malaria can then visually be diagnosed under the microscope [2].

Thin blood smear procedure is quick to perform and has a detection-limit of 50-100 parasites/µl blood. After an intensive training the performer should be capable to distinguish various *Plasmodium* species [4, 55]. For a thick smear a more intensive training is needed, but it has a better detection-limit of 4-20 parasites/µl blood than the thin smear [4, 48, 55]. It might be easier to screen the slide, but it is more difficult to distinguish between *Plasmodium* species in mixed infections, especially between species at trophozoite stage [4].

3.2 Rapid Diagnostic test

As an alternative to microscopy the Rapid Diagnostic test (RDT), Dipstick method or Malaria Rapid Diagnostic Device (MRDD) was designed. RDT is based on a lateral flow immune-chromatographic Ab-detection test. This implies that it studies the presence

of parasite Ags present in human blood with the use of polyclonal or monoclonal dye-labeled Abs [4, 56]. In contrast to microscopy with a detection-limit of 4-20 parasites/µl blood [4, 48, 55], RDT has a detection-limit of about 100-200 parasites/µl blood (*P. falciparum*) and > 500 parasites/µl blood (*P. vivax*) [56]. This makes the RDT test less sensitive. However, as the test is easy to perform directly on the field (15-30 minutes after a finger-prick blood collection) it has a great value as point-of-care test [57]. At places where no microscopes are available it is a frequently used method that contributes greatly to early diagnosis and subsequent treatment as it can be performed in the rural areas itself [4, 56], and by village malaria/health volunteers.

For this test, human blood is mixed with a lysing agent. This helps to rupture the erythrocyte to release more parasite Ags. Then a dye-labeled Ab (target Ab) is added and binds with the Ag. This Ab-coated-Ag passes a nitro-cellulose strip through capillary flow. When the Ab-coated-Ag crosses the test and control line another line will become visual when a parasite-dye-labeled Ab binds to the target Ab [56].

3.3 Molecular diagnostic tools

Other malaria detection methods are based on molecular diagnostics that involve detection of specific DNA regions within the parasite genome. Compared to the conventional methods, these techniques have increased sensitivity and specificity to detect malaria parasites in low parasitaemia or mixed infections [58]. Most frequently used methods are different types of the *Plasmodium* specific PCR assays, such as nested PCRs [48, 59], real-time PCR assays based on fluorescence dyes [59], multiplex RT-PCRs [58] and Loop-mediated isothermal amplification (LAMP) assays [58]. These *Plasmodium* specific PCR assays can detect as few as 0.2 parasites/µl blood [59], which is a big step forwards compared to approximately 4-100 parasites/µl blood with microscopy or RDT [2, 48].

In addition, PCR can be useful in studies on drug-resistant parasites, mixed infection and can be used to process large numbers of samples when automated [2]. However, despite these advantages, it is also a complex technique that requires special training, with high equipment and consumable costs [58]. As a result, it is difficult to perform PCR routinely in developing countries, and as such in its current form it cannot be used a point-of-care test [2].

4. Serological approaches

For confirmation of parasite exposure serological tests can be used [2, 4]. However, serology cannot be used in the acute diagnosis of malaria in contrast to the malaria

parasite detection methods mentioned in the previous paragraphs, but can be very useful in measuring previous malaria exposure [60]. During the recent years, serology became a valuable tool in obtaining epidemiological information in malaria control programs. As Abs remain in the blood for a longer period (a few months to a few years) than the parasite, serology could be more sensitive in measuring host-parasite contact (Figure 1-4). Currently the debate on longevity of Abs remain controversial [46, 53, 61–66]. Therefore, a better knowledge of the persistence of Abs could be useful to distinguish a recently infected individual from those infected months or years before.

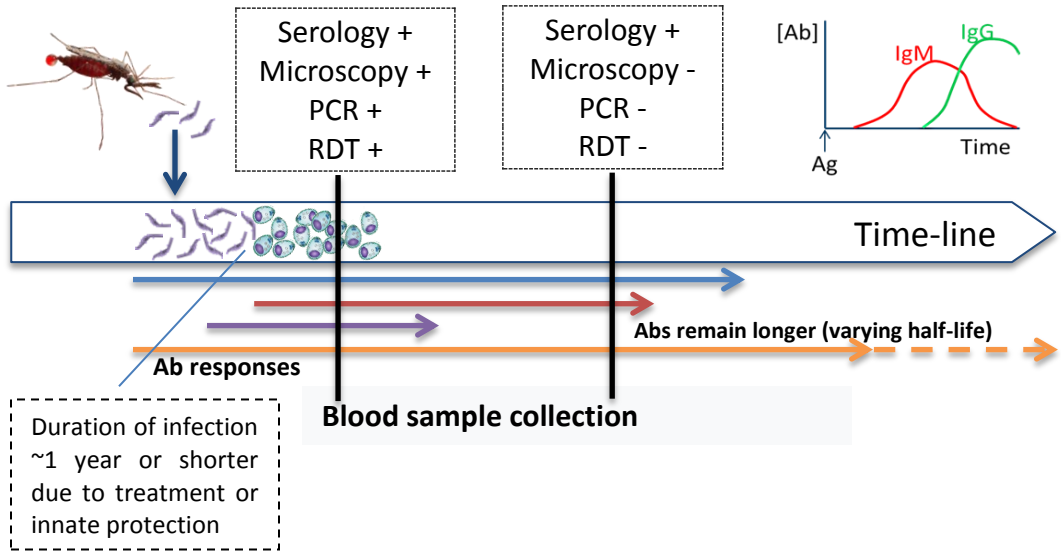

Figure 1-4: Serological markers might be more informative in estimating the Force of Infection (FOI) by sero-conversion rate (SCR) in pre-elimination settings as compared to classical parasite detection methods as they can be used to estimate recent or past malaria exposure, depending on the used markers and their longevity. When mosquitoes bite an individual, Ab-responses will be triggered by the immune system. These Ab-responses (coloured lines) can vary in length between short and long-term Ab-responses. When a blood sample of this individual is collected and PCR is performed on that blood sample the present parasites will be detected and this will give only a snapshot of the endemicity, while serological methods are based on the detection of Abs against Ags of malaria parasites they will provide a picture of the endemicity over a larger period. This will give us information about the FOI. (Source IgM/IgG graph: http://www.medicine.mcgill.ca/physio/vlab/immun/backg.htm)

4.1 General background on malaria immunology

The vertebrate host immune response elicited by the bite of an infectious *Anopheles* mosquito is highly complex [67]. Especially, because people living in malaria endemic countries are usually repeatedly infected with malaria parasites and acquire immunity gradually [68]. Sporozoites inoculated into peripheral circulation trigger an immune

response regulated by the innate and adaptive immune responses [13, 67, 69–71]. Both types of immune response are involved in memory buildup, but the large variety of underlying mechanisms to neutralize the parasite are still poorly understood [67].

The innate immunity (first line defense) is mainly regulated by dendritic cells, natural killer cells (NKs), NK-T-cells, Kuppfer cells, γδ-T-cells and macrophages. This arm of the immune system stimulates and regulates the adaptive immune response via production of specific cytokines, ultimately resulting in Ag-specific humoral- and cell-mediated responses [67].

Previous studies show that the humoral immune system appears to play a major role in anti-parasite immunity [72–74]. As the aim of this thesis is to develop an assay that detects changes in the force of malaria infection on both short and long term, the focus in the following paragraph will be on the Ab-responses and B-cells.

4.2 Antibodies produced against different life stages of malaria

Dendritic cells belong to the antigen-presenting cells, present in the skin-associated lymph nodes. They take up sporozoites and present peptide fragments to the $CD4^+$ and $CD8^+$ T-cell [67]. The function of the $CD4^+$ T-cells is based on the lymphokine production in Th1 (IFN-y production) and Th2 (IL-4 and IL-5 production) cells [75]. The $CD4^+$ T-cells will be activated and secrete pro-inflammatory cytokines, which subsequently activate the B-cells [67].

After a first exposure of sporozoites in the peripheral circulation, B-cells (Figure 1-5) present in the bone marrow or to a lesser extent in the spleen are triggered to respond [76]. B-cells display various Ag-receptors (membrane-bound immunoglobulins (Ig)) at their surface that are capable of capturing Ags. When a receptor has captured an Ag these B-cells rapidly undergo clonal expansion, and subsequently differentiate into plasma cells and memory B-cells (MBCs). There are two types of plasma cells, short-lived and long-lived plasma cells [76]. Evidence indicates that the short-lived plasma cells remain in the lymph nodes and primarily secrete IgM, while long-lived plasma cells together with MBCs migrate to the bone marrow and secrete IgG and IgA [70, 72, 77]. IgM is associated with the presence of the parasites and not related to the protective immunity [72]. In order to define the transmission intensity, the IgM prevalence is of limited value as it only remains in the human host for a short period (several days to a month, Figure 1-4) [46, 78]. IgG Abs are associated with protective immunity and replace the IgM when the parasitic load decreases (Figure 1-4). There are four subclasses of IgG. They are classified according to their IgG affinity to Fc-

receptors and the complement and effector cell activation. Besides, they are also classified by their IgG amount present in the blood circulation and are considered as age varying. IgG responses dominated by IgG1 and IgG3 are related to the malaria protection, whereas IgG2 and IgG4 are not related to malaria protection [70, 72].

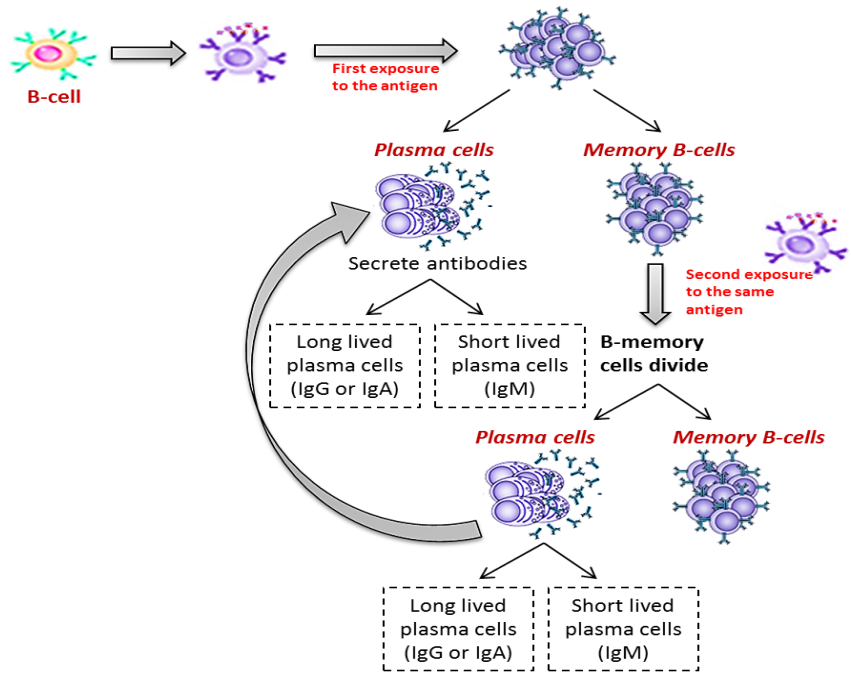

Figure 1-5: Process of memory build-up. B-cells are present in the bone-marrow. They have different Ag-receptors that, in the primary immune response, try to bind to Ags present on the parasites. When an Ag-receptor captures an Ag, B-cells divide rapidly, after which they start to produce memory B-cells and plasma cells. After which plasma cells immediately attack the parasite. With a secondary infection the present memory B-cells will recognize the Ags. Plasma cells that already exist will respond instantly to the parasite, while at the same time the MBCs will rapidly divide and produce new plasma cells. As a result the amount of memory B-cells and plasma cells increase with Age, due to repeated infection. (Source: NA)

During a second exposure present MBCs immediately undergo clonal expansion, after which they differentiate into new plasma cells (Figure 1-5) [63, 76]. Due to recurrent exposure the amount of plasma cells and MBCs increase with age [77]. Then, in the meantime the present plasma cells (long-lived plasma cells) secrete IgG-Abs that respond to the sporozoitic Ags. IgG-Abs present in the skin focus on the circumsporozoite protein (CSP) [79] and *falciparum* 13 (Pf13) (Figure 1-6) [80, 81]. These IgG-Abs inhibit subsequent sporozoite invasion of the hepatocytes. They do not completely stop the sporozoite entry [67, 79], causing some sporozoites to migrate to the hepatocytes in the liver, while the sporozoites in the skin are drained to the nearest lymph nodes [67].

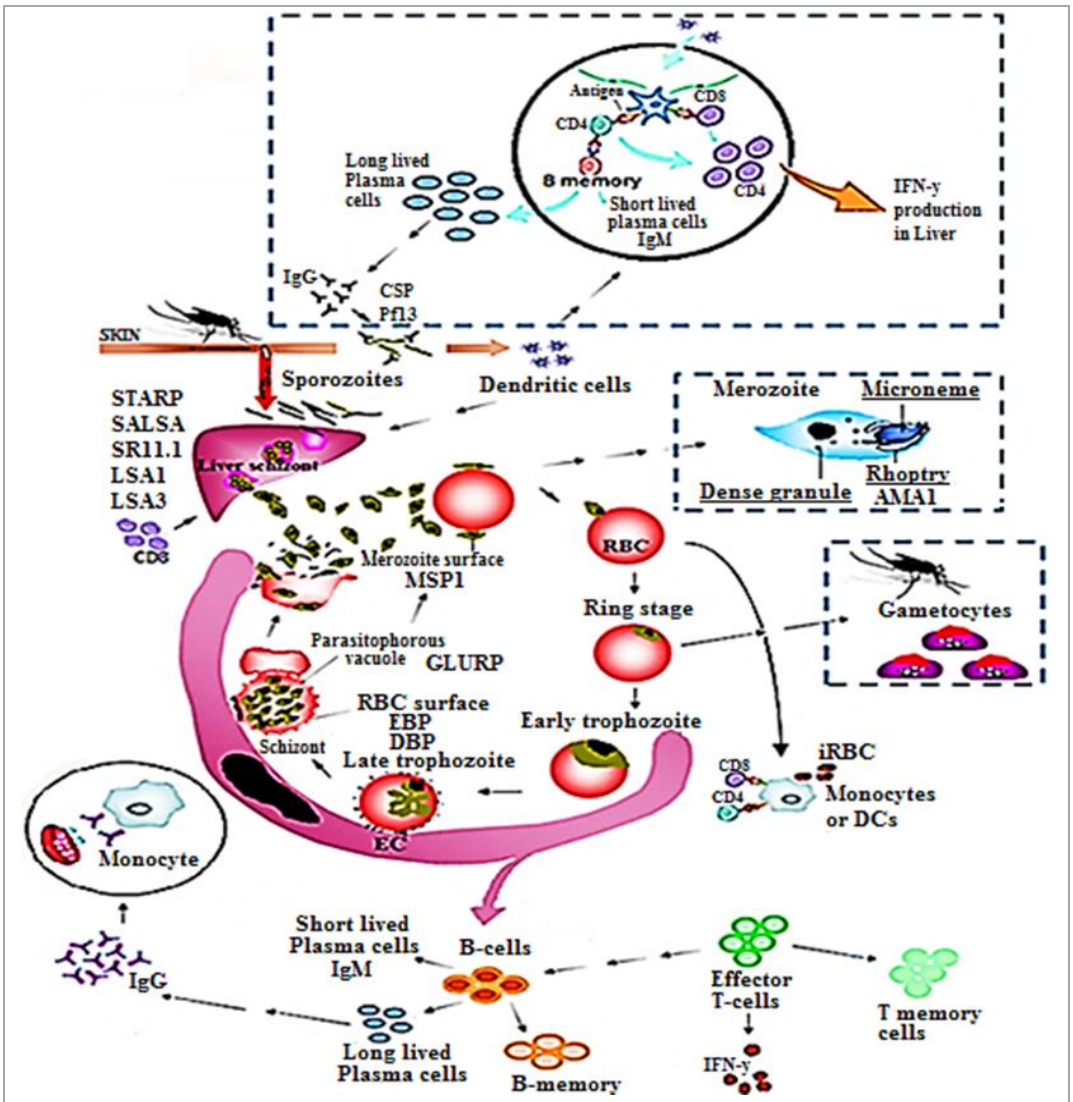

Figure 1-6: Representation of the serological markers in the sporozoite, liver and blood-stages of the *Plasmodium* life-cycle [67, 82]**. Regulation of the immune response is elicited through the bite of an infectious *Anopheles* mosquito. B-cells present in the bone marrow are the first to respond to an invasion of *Plasmodium* parasites. These B-cells possess various Ag-receptors to capture Ags. After capturing, B-cells undergo clonal expansion and differentiate into short-lived plasma cells (PCs) and memory B-cells (MBCs) to eventually establish memory. Short-lived plasma cells secrete IgM-Abs to destroy the parasite, while MBCs remain inside the human host for a longer period, by producing long-lived PCs. Long-lived PCs secrete IgG-Abs, which can subsist for months after infection. Due to recurrent infection and past exposure the amount of PCs and MBCs increase with age.**
(Adapted from multiple existing figures: The erythrocytic cycle is derived from Bolad *et al.* [82]**, while the memory build up is derived from Douradinha *et al.*** [67]**).**

In the meanwhile the $CD4^+$ T-cells trigger the dendritic cells for $CD8^+$ T-cells activation. These $CD8^+$ T-cells differentiate into CD8 effector T-cells and migrate to the liver, where they interact with the infected hepatocytes and secreted IFN-γ (Interferon

gamma) [67]. IL-12 is a cytokine known to play an important role in the early responses against *Plasmodium*, and is closely linked to or acts through IFN-γ production, allowing an early and sustained Th1 response [75]. Both IFN-γ and IL-12 can stimulate the production of endogenous nitric oxide (NO) to kill the parasite (Figure 1-6) [67]. The sporozoite and liver stage specific IgG Abs generated by the long-lived plasma cells focus on sporozoite threonine- and asparagine rich protein (STARP), sporozoite- and liver stage antigen (SALSA) and sub-region antigen SR11.1 [52, 83, 84]. In addition, these Sporozoite and liver-stage IgG Abs are also directed against liver-stage antigens (LSA), which are only expressed inside the liver [85, 86].

As indicated IFN-γ will be secreted by the CD8 effector T-cells and can inhibit parasite development. However, this is frequently insufficient, and as a result merozoites still emerge from the liver to invade the RBCs [63, 67]. They replicate and burst out of the infected RBCs to invade new RBCs. During the blood-stage of the parasite, different types of antigen presenting cells present parasite peptide fragments from infected RBCs to activate $CD4^+$ and $CD8^+$ T-cells [67]. Fusion of the *P. falciparum* parasite and the dendritic cells stimulate the effector T-cells [67, 73] to differentiate into effector memory cells over time. The latter stimulate the B-cells for development of short- and long-lived plasma cells as well as memory B-cells [67]. The long-lived plasma cells are just as described before capable of secretion of IgG Abs for long periods of time.

The IgG-Abs against merozoites from erytrocytes include Abs targeted against merozoite surface protein (MSP), apical membrane antigen (AMA1) [87], and contribute to inhibit a merozoite invasion of erythrocytes, intra-erythrocytic growth and to improve removal of infected erythrocytes from the circulation by attaching to the erytrocytic surface [70, 87]. Duffy binding protein (DBP [88]) is related to the homologous erythrocyte-binding proteins (EBP [89]) and is only expressed on the merozoite surface of *P. vivax* in infected individuals. Anti-DBP-Abs play a crucial role in the prevention of erythrocyte invasion by blocking DBP-binding to the Duffy Ag-receptor [90]. Finally, the glutamine-rich protein (GLURP) is a highly antigenic exo-antigen (separable from its source), expressed at each stage in the *P. falciparum* life cycle in the human host, as well as on the surface of the newly released merozoites [52, 91]. All Ags discussed are used in this study to provide information about parasite exposure at community level.

4.3 detecting anti-Plasmodial antibodies

Since the 1960s, serological methods are based on the indirect immunofluorescence assay (IFA test) and Enzyme Linked ImmunoSorbent Assay (ELISA) (Figure 1-7). Until

now, the IFA test was the reference for malaria Ab determination and has been mainly used in epidemiological studies [2, 92, 93].

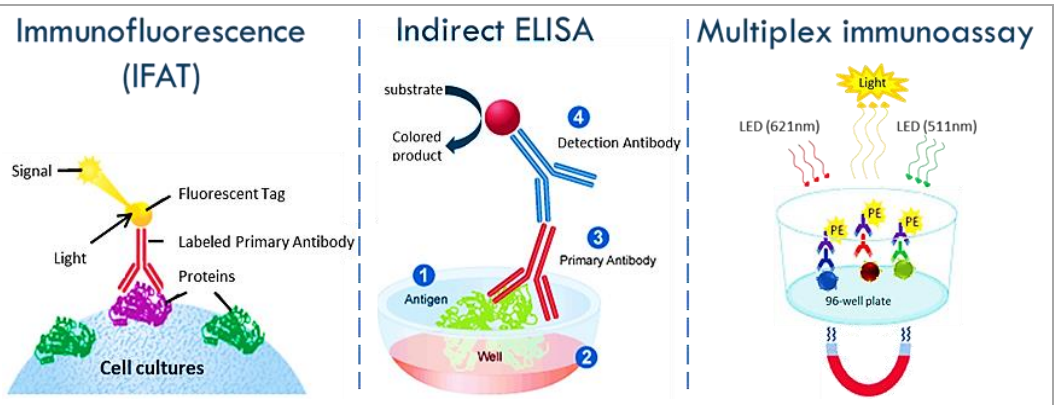

Figure 1-7: Differences between the most commonly available serological tools [94, 95]. The IFA test uses the whole parasite as an Ag and is visually observed under a fluorescent microscope [95, 96], while an ELISA uses a single Ag that is fluorescent labelled for detection [97]. A multiplex immunoassay is based on the same principle as an ELISA, but can measure multiple Ags simultaneously [95].
(Source: Multiplex immunoassay is adapted from the Luminex Coorporation [95]).

The principle of the IFA test is that Abs present in blood samples are tested for their ability to react with whole parasite [96]. IFA is performed on infected blood dried on a microscope slide. The slides are then covered with a serial dilution of test serum and with a fluorescent labelled anti-globulin complex [98]. The IFA test is considered a standard diagnostic Ab detection test due to its simplicity to perform as well as its high specificity and sensitivity. Nevertheless, it is also time-consuming and the interpretation of final results may derive in subjective analyses and conclusions. Therefore, the ELISA technique is preferred for large epidemiological studies when mass screening of blood samples is needed [97], as interpreting results is more accurate [98]. The difference between the ELISA and IFA is that in the ELISA a single Ag is used instead of the whole parasite and the anti-Ab specific globulin forms a complex with an enzyme or colorant that change the substrate into a detectable state [99].

In order to improve these tests, new techniques, such as multiplex bead based-assays have been developed. Using this technique we can detect multiple Ags in a large population, so that it can be used as high throughput screening. It combines microspheres (polystyrene paramagnetic beads), lasers and a detection system to measure more analytes at the same time [100, 101]. The MAGPIX is a small compact device, and its procedure is based on an ELISA. The main difference with an ELISA is that Ags are coupled to the surface of the beads instead of to the well [52]. This makes

it possible to multiplex the different beads into one assay to capture and quantify various Abs in one blood sample [52]. Each bead is labeled with a different color code based on a specific ratio of two fluorochromes that can be detected by the three read-out systems. This technique turned out to be quicker, more precise, sensitive and reproducible than the tradition serological methods.

4.4 Assessing serological data

In epidemiological studies it is important to mimic the transmission of the malaria disease, identify the key factors of transmission and inform the underlying process that drives that transmission dynamics. In order to achieve this, researchers routinely used mathematical models to give a simplified representation of the complex reality. The biggest variety of models capable of representing the dynamics of malaria transmission is designed for parasitological, entomological, clinical and epidemiological data. However, in case of serological data modelling is still in its infancy. Therefore, this paragraph focuses on the statistical approaches and mathematical models used in this thesis for serological data.

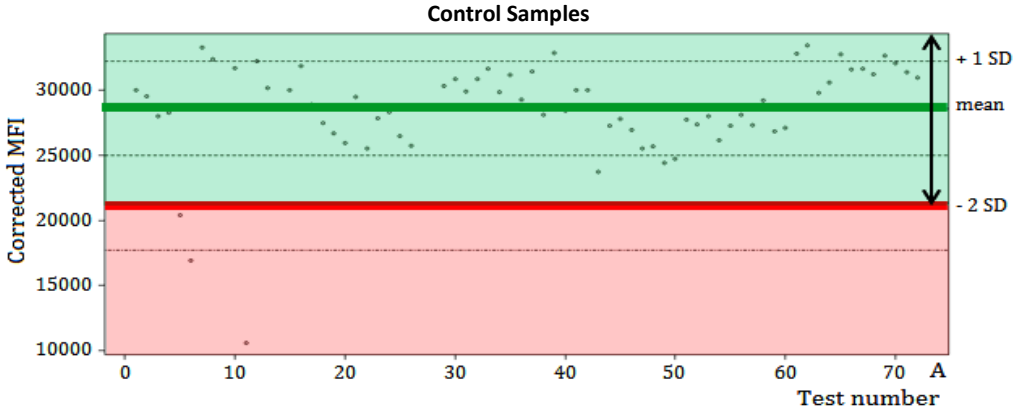

Figure 1-8: Example Levey-Jenning Chart. The dots represent the control samples per plate. If these dots fell out of the two standard deviation (red area), the plates belonging to these control samples were rejected and re-analysed.
(Source: Kerkhof *et al.* [102]).

A first step before the serological data can be assessed is performing a quality control to detect, reduce and correct deficiencies occurred during the laboratorial process prior to the outcomes of the results. This step is an important measure of precision, showing how well the measurements reproduce similar results over time under varying laboratorial conditions [103]. Interpretation of the serological data involves both graphical and statistical methods of which 'Levey-Jenning charts' (Figure 1-8)

provide the most easily visualized method. In a Levey Jenning chart the x-axis represent the amount of control sample used and the y-axis represent the outcome measurement. On the y-axis the mean-value and the one, two and three standard deviations are marked. These are used to accept or reject the control values [104].

After quality control, the serological data can be further explored to finally investigate the malaria transmission. For this, the most widely used model is the catalytic model, developed by Muench *et al.* [105] and introduced to malaria by Draper *et al.* [106] as the 'constant infection rate model'. As the name already suggests, this model assumes only one single seroconversion and seroreversion rate for all different age groups in a population during the wet season. This means that naïve and seropositive people return to being seronegative (Figure 1-9) with a constant force of infection rate [46]. The force of infection rate corresponds to the incidence of seroconverting and is a representation of the natural measure of transmission intensity, and corresponds to the number of infections acquired per person per year [41].

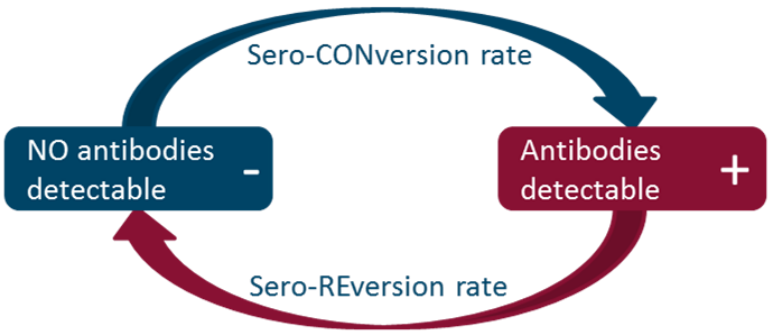

Figure 1-9: Flow diagram for catalytic model.
(Source: NA).

In the first models the titre data was converted into prevalence data by using negative sera from Europeans that were never exposed to malaria. These sample outcomes were used to define a threshold. However, the variety in genetics and different exposure to various local pathogens resulted in the fact that these controls were not appropriate [46, 107, 108]. Therefore, an alternative threshold approach, based on a mixture model, was used. In this model, the positivity per antigen is defined as a measurement more than three standard deviations above the mean measurement of the negative distribution [62]. Eventually, the outputs of such a model can provide a proxy for malaria transmission intensity [50]. However, Abs can persist for many years and people might stay seropositive for a while. By seroconverting data the estimated

force of infection rely on the choice of the threshold between the negative and positive distribution, which lacks standardisation and might bias the outcomes. To that extent, a binary structure does not represent the actual fluctuations in Ab-levels, in which the Ab-response might do reflect changes in transmission [41]. Therefore, the threshold model is only used in chapter 4 to see if the implementation of the serological assay succeeded. In the course of this thesis, the emphasis laid on the use of linear mixed models, which have been conducted directly on the raw continuous data.

A linear mixed model is a model that includes both fixed (binary data) and random effects (continuous data) [109]. This model describes a straight line, based on the specific normally distributed data points useful in a setting where measurements are made on clustered nature of the study design [110, 111]. One of the advantages compared to the more traditional methods (e.g. repeated measures ANOVA) is that it deals with missing values [111]. When working with non-normally distributed data a generalized linear mixed model is used. This is an extension to the idea of the linear mixed models [112] and commonly used on population level data [113].

4.5 Studies performing serological multiplex assays

Serology based on ELISA is widely used to estimate malaria transmission. The ELISA is used to screen serum samples for the presence of Abs to different Ags. Different serological studies performed in the Greater Mekong Subregion indicate large variation in sero-prevalence in Thailand [114–119], Malaysia [120–122] and Myanmar [123]. These outcomes depend on which Ag tested. In the high transmission areas of Cambodia approximately 2-5% tested positive for anti-*P. falciparum* Abs and approximately 6-8% tested positive for anti-*P. vivax* Abs [50].

For testing more Ags at the same time multiplex assays can be used. Although there is record of previous studies performing such multiplex assay, the Greater Mekong Subregion has remained unexplored. Table 1-1 displays several multiplex assay studies performed in different areas in Africa. This suggests that multiple Ags can be tested at the same time instead of one. The results indicate that with GLURP (98%), SALSA2 (96%), CSP (96%), LSA1-41 (87%) and STARP-R (80%) the highest sero-prevalence can be achieved. Medium seroprevalence rates are found with SR11.1 (64%), LSA1-J (58%), SALSA2 (58%) and GLURP-P3 (53%). The other Ags result in lower seroprevalence rates ranging between 0-50 percent. However, as human and parasite populations between regions or continents might be different, it is important that the antigenicity of the Ags is tested in the population of interest [53].

Table1-1: Multiplex studies performed in other parts in the world on the same Ags as used in this thesis.

Country	Year	*Plasmodium* species	Antigens	Age (years)	Total # of samples	Positive samples	Sero-prevalence (%)	REF
Haiti (Léogâne)	2014	*P. falciparum*	PfMSP1-19* (longitudinal data)	< 6	142	25	11	[65]
Haiti (Miton)			PfMSP1-19* (cross-sectional data	0.2 - 90	383		14	
Senegal (Ndiop village)	2014	*P. falciparum*	Pf13*	< 5	11	2.3	18	[124]
				5 – 10	39	8.7	59	
				10 – 14	34	12.5	12.5	
				15 – 29	65	11.1	11.1	
				>30	68	8.6	8.6	
			PfMSP1-19*	< 5	11	3.4	36	
				5 – 10	39	3.7	33	
				10 – 14	34	5.9	59	
				15 – 29	65	6.5	57	
				>30	68	6.9	69	
Mozambique	2013	*P. falciparum*	PfAMA1* (3D7 strain)	0 – 24	28	19	68	[125]
			PfAMA1* (FVO strain)	months	28	11	39	
			Pf MSP1-42* (3D7 strain)		28	23	82	
			Pf MSP1-42* (FVO strain)		28	15	54	
			PfAMA1* (3D7 strain)	travelers	20	9	45	
			PfAMA1* (FVO strain)		20	6	30	
			Pf MSP1-42* (3D7 strain)		20	10	50	
			Pf MSP1-42* (FVO strain)		20	8	40	
Northern Senegal (June)	2004	*P. falciparum*	LSA1-41	1 – 8	59	7	11.9	[126]
			SALSA1		59	14	23.7	
			GLURP		59	31	52.5	
			LSA1-J		59	10	16.9	
			LSA3-NR2		59	9	15.2	
Northern Senegal (Sept)			LSA1-41	1 – 8	62	12	19.3	
			SALSA1		62	10	16.1	
			GLURP		62	25	40.3	
			LSA1-J		62	11	17.7	
			LSA3-NR2		62	16	25.8	
Northern Senegal (Dec)			LSA1-41	1 - 8	65	17	26.1	
			SALSA1		65	12	18.5	
			GLURP		65	32	49.2	
			LSA1-J		65	13	20.0	
			LSA3-NR2		65	16	24.6	
Republic of Djibouti	2002	*P. falciparum*	LSA1-41, LSA1-J, LSA3-NR2, GLURP, GLURP-P3, SALSA1, SALSA2, TRAP1, STARP-R, CSP, SR11.1, PfMSP1-19* and PfAMA1*.	15-45	1910	602	31.5 (29.4 – 33.7)	[127]
		P. vivax	PvMSP1-19*, PvMSP1-42*			334	17.5 (15.8 – 19.3)	
Senegal (travelers)	1995	*P. falciparum*	LSA1-41	>20	124	7	5.6 (2.3 – 11.3)	[52]
			LSA1-J		124	4	3.2 (0.9 – 8.1)	
			LSA3-NR2		124	1	0.8 (0.0 – 4.4)	
			LSA3RE		124	0	0.0 (0.0 – 2.9)	
			GLURP		124	5	4.0 (1.3 – 9.2)	
			GLURP-P3		124	1	0.8 (0.0 – 4.4)	
			SALSA1		124	0	0.0 (0.0 – 2.9)	
			SALSA2		124	2	1.6 (0.2 – 5.7)	

				TRAP1		124	0	0.0 (0.0 – 2.9)
				TRAP2		124	0	0.0 (0.0 – 2.9)
				STARP-R		124	1	0.8 (0.0 – 4.4)
				CSP		124	7	5.6 (2.3 – 11.3)
				SR11.1.		124	1	0.8 (0.0 – 4.4)
				SALIV 1		124	0	0.0 (0.0 – 2.9)
				SALIV 2		124	1	0.8 (0.0 – 4.4)
Senegal	**(Diama**	1995	*P. falciparum*	LSA1-41	>20	38	19	50 (33 - 67)
village)				LSA1-J		38	4	11 (3 - 25)
				LSA3-NR2		38	3	8 (2 - 21)
				LSA3RE		38	0	0 (0 -9)
				GLURP		38	25	66 (49 - 80)
				GLURP-P3		38	7	18 (8 - 34)
				SALSA1		38	7	18 (8 - 34)
				SALSA2		38	16	42 (26 - 59)
				TRAP1		38	0	0 (0 - 9)
				TRAP2		38	0	0 (0 - 9)
				STARP-R		38	3	8 (2 - 21)
				CSP		38	10	26 (13 - 43)
				SR11.1.		38	9	24 (11 - 40)
				SALIV 1		38	3	8 (2 - 21)
				SALIV 2		38	0	0 (0 - 9)
Senegal	**(Ndiop**	1995	*P. falciparum*	LSA1-41	>20	40	33	83 (67 - 93)
village)				LSA1-J		40	15	38 (23 - 54)
				LSA3-NR2		40	7	18 (7 - 33)
				LSA3RE		40	0	0 (0 - 9)
				GLURP		40	39	98 (87 - 100)
				GLURP-P3		40	21	53 (36 - 68)
				SALSA1		40	23	58 (41 - 73)
				SALSA2		40	23	58 (41 - 73)
				TRAP1		40	3	8 (2 - 20)
				TRAP2		40	0	0 (0 - 9)
				STARP-R		40	15	38 (23 - 54)
				CSP		40	32	80 (64 – 91)
				SR11.1.		40	12	30 (17 - 47)
				SALIV 1		40	2	5 (1 - 17)
				SALIV 2		40	0	0 (0 - 9)
Senegal	**(Dielmo**	1995	*P. falciparum*	LSA1-41	>20	45	39	87 (73 - 95)
village)				LSA1-J		45	26	58 (42 -72)
				LSA3-NR2		45	15	33 (20 - 49)
				LSA3RE		45	3	7 (1 - 18)
				GLURP		45	45	100 (92 - 100)
				GLURP-P3		45	15	33 (20 - 49)
				SALSA1		45	24	53 (38 - 68)
				SALSA2		45	43	96 (85 - 99)
				TRAP1		45	2	4 (1 - 15)
				TRAP2		45	1	2 (0 - 12)
				STARP-R		45	36	80 (65 - 90)
				CSP		45	43	96 (85 - 99)
				SR11.1.		45	29	64 (49 - 78)
				SALIV 1		45	5	11 (4 - 24)
				SALIV 2		45	0	0 (0 - 8)

***Recombinant proteins**

5. Highlighting malaria in the Greater Mekong Subregion and Cambodia

The fieldwork for this thesis was carried out in Cambodia, which is part of the Greater Mekong Subregion. Therefore in the next paragraphs an overview of malaria in the Greater Mekong Subregion and in particular Cambodia is given.

5.1 Geographical situation in the Greater Mekong Subregion

The Greater Mekong Subregion comprises Cambodia, People's Republic of China (Yunnan province and Guangxi Zhuang Autonomous Region), Lao People's Democratic Republic, Myanmar, Thailand and Vietnam [3, 128]. The malaria burden in this region, is high and impacts socioeconomic development [129]. However, over the past 15 years the situation has greatly improved which is reflected in a significant decrease in annual malaria incidence and mortality rates. However, this region still faces challenges in controlling malaria, due to the enormous geographical heterogeneity. This manifests itself in a varying malaria burden per country, and high malaria transmission (human parasite contact) along the borders and in forested areas.

The Greater Mekong Subregion (2.4 million km²) has a total population of 278 million people of which 450,000 confirmed malaria cases in 2013 [129]. Between 2000 and 2013 a decrease in malaria mortality and morbidity of 35% was seen, as well as a 30% reduction in the annual number of malaria deaths [129]. The highest malaria burden is observed in Myanmar that accounts for 77% of the confirmed malaria cases and 79% of the malaria death [129]. Furthermore, Myanmar is also the country in the Greater Mekong Subregion that had the highest decrease in incidence during the past years. Moreover, malaria cases are decreasing in Cambodia and Thailand, while in Lao PDR since late 2011 a malaria resurgence took place resulting in an increase in reported malaria cases [129, 130].

Table 1: Malaria situation in the Greater Mekong Subregion countries in 2014 [129]**.**

Country	Total population (mil.)	Population at risk (mil.)	Population at high risk (mil.)	Confirmed malaria cases	Malaria deaths	Incidence of malaria cases per 1000 pop.
Cambodia	15.3	10.8	7.36	26 278	18	1.7
Lao PDR	6.7	6.2	2.1	48 071	4	7.2
Myanmar	53.4	31.7	8.4	152 195	92	2.9
Thailand	67.7	33.8	5.4	37 921	38	0.6
Vietnam	92.4	68.1	6.3	27 868	6	0.3

The kingdom of Cambodia (181,000 km²) is bound in the north by Thailand and Lao PDR, on the east and southeast by Vietnam and on the west by Thailand and the Gulf of Thailand. One of the greatest geo-physical features is the Mekong River, which places Cambodia in the Greater Mekong Subregion. Cambodia has a tropical climate

with seasonal malaria transmission, due to the rainy season from late April till early October. The transmission is highly heterogeneous, focal, and unstable in the north, west and northeast regions of Cambodia that results in a variation in malaria incidence within small areas or populations [35, 131].

5.2 Biology of malaria in Cambodia

Nowadays, malaria is still a major cause of mortality and morbidity with highest impact on ethnic minorities, temporary migrants, people living in forested areas, and plantation workers. Divided over 15,388 villages and 15.3 million people, approximately 71% of the people are at risk [132], and about 48% lives in the highly endemic forested areas [4, 44]. In the past, *P. falciparum* was the predominant parasite species in Cambodia, while *P. vivax* increased between 2006 and 2012 [132]. In 2012 the two main *Plasmodium* species that account as culprits for malaria were *P. falciparum* (37%) and *P. vivax* (48%) and in 15% of the cases they were found as mixed infections [132]. In Cambodia malaria is transmitted by one of the following vector species: *Anopheles dirus, Anopheles minimus*, *Anopheles maculatus*, *Anopheles barbirostris* [131, 133].

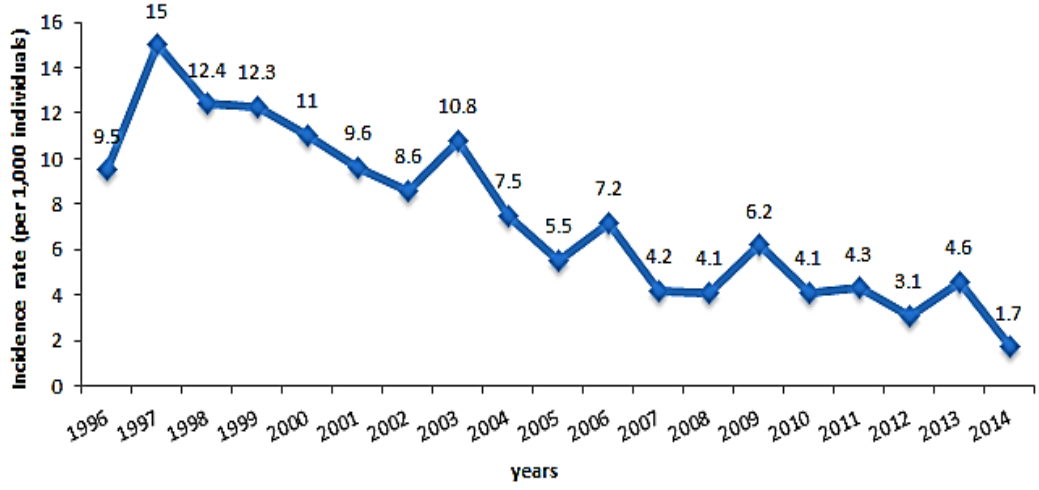

Figure 1-10: Annual malaria incidence rates (IR per 1,000 individuals) in Cambodia starting from 1996. Data from 1996 – 2004 [131]**, 2000 – 2011** [134]**, 2012** [135]**, 2013** [136] **and 2014** [19] **.**

5.3 Elimination strategies in Cambodia through the years

The efforts to control malaria date back to 1951, when the Ministry of Health (MoH) started with a malaria control program supported by the WHO. The program was based on malaria treatment and vector control, and proved to be successful until the Khmer Rouge invaded Cambodia in 1971. Under the leadership of Pol Pot, the Khmer

Rouge posed a regimen characterized by civil wars and brutal genocide [131, 137]. They deported most of the Cambodian population, especially health workers and teachers, to high endemic areas and about two million people died from violence, malnutrition and lack of imported medicine leading to deaths from diseases like malaria [131, 138]. The Khmer rouge regime lasted from 1975 till 1979 when Vietnam put an end to it. This was the start of a socialistic regime after which the country was excluded by the western international support. As a result, Cambodia was left with an underdeveloped infrastructure and capacity, especially in the rural areas. As of 1997 the situation became more stable and Cambodia underwent a remarkable rebound, after receiving international support from the United Nations [138].

In the eyes of the international organizations the border between Thailand and Cambodia still remained a problem for malaria. Since the 1960s this border is frequently an epicenter of emerging resistance of *P. falciparum* to antimalarial drugs [139]. For that reason, in 2010 the WHO, Thailand's government and the Cambodian government's National Centre for Parasitology, Entomology and Malaria Control (CNM) developed an epidemiological tool named 'Focused Screening and Treatment' (FSAT) [139]. FSAT is a PCR based strategy used for the detection of asymptomatic parasite carriers and has been developed in an attempt to eliminate multi-drug resistant (MDR) *P. falciparum* parasites at the Pailin province, the so-called 'elimination' area along the border between Thailand and Cambodia [139]. Between 1997 till 2012 (Figure 1-10), due to a successful national anti-malaria program, authorities reported a decrease in the annual parasite incidence rate of approximately 79% (microscopically confirmed cases) [131, 134, 135]. The national anti-malaria program has been set up by the ministry of health and the CNM, with the focus on creating a Cambodia that is totally free form the burden of malaria [131]. It has a partnership with the WHO and the Bill & Melinda Gates Foundation. This national program bases its malaria control on two major pillars: (1) vector control and (2) rapid diagnose and treatment.

5.4 Vector control

As already discussed in the previous paragraph vector control is a key element in the fight against malaria, with the focus on reducing the burden of malaria by putting a halt to parasite transmission from host to mosquito and back. Over recent years vector control interventions have been applied worldwide and since 2000 Cambodia started with a free distribution of ITNs [3]. In 2007 they started with the distribution of long-lasting insecticidal nets (LLINs) that would be effective for at least three years.

From 2012 on, a one-net-to-one-person ratio was aimed at, spectacularly increasing bed net coverage.

5.5 Rapid diagnosis and treatment

In Cambodia the case management policy for malaria drafted in 2000 by the Ministry of Health and was amended in 2010 [3, 131]. From 2001 Cambodia started with the use of "Village Malaria Workers" in 30 different villages in Rattanakiri, a province with a high malaria transmission. VMWs are volunteers who are appointed in a community consensus, 2 VMWs per village who receive an incentive of $4 per month. They are trained to determine the malaria diagnose through RDTs on any suspected of having malaria. They are also assigned to only provide treatment to positive cases with antimalarial drugs following the national guidelines. The VMWs are monthly supervised and re-supplied by the provincial malaria staff [140]. Treatment is based on ACTs, because of rapid resistance of the malaria parasite in some areas [59], and Cambodia was the first country that added ACTs into the national policy against malaria [140].

From 2009 a scale-up of the VMW program to cover 300 villages took place, with support from the WHO and the Global Fund [140]. In addition, the VMWs were also trained to recognize and treat other base diseases, such as diarrhea and respiratory infections. As a result of the rapid and accessible diagnosis and treatment, rural communities gain more access to diagnosis and treatment that leads to a decrease in malaria cases [34].

6. Application of the background information into this PhD-thesis

The Ab-levels against 21 Ags (as discussed in paragraph 4) are analyzed during this PhD-thesis to provide information about parasite exposure at community level. The acquired information about their place inside the immune system will be taken into account during all research approaches. The variety of the 21 Ags used in the present thesis consists of peptides and recombinant proteins representing different life stages of the parasite and different *Plasmodium* species (*P. falciparum* (11 Ags), *P. vivax* (7 Ags), *P. malaria* (1 Ag), and peptides specific for *Anopheles gambiae* salivary protein (2 Ags)). The peptide selection is based on a previous study performed by Ambrosino et al. [52], and the recombinant proteins on the availability at the 'Institut Pasteur' (IP) Paris. It is important to keep in mind that these 21 Ags are not the only existing Ags, but the most routinely used Ags for this purpose. A prime example is a recent study performed by Helb *et al.* [53], who evaluated 856 *P. falciparum* Ags of 186 Ugandan children. These 856 Ags elicit Ab-responses with different magnitudes and kinetics,

resulting in a diverse set of potential biomarkers for exposure [53]. In contrast the Ags used in the presented PhD project are not only directed against *P. falciparum* Ags. Additionally, the current thesis uses 11 *P. falciparum* Ags, along with three additional Ags (Pf13, SR11.1 and SALSA2) that were not included in the study of Helb *et al*. [53]. Furthermore, Ags specific for *P. vivax, P. malariae* and Ags specific for the *Anopheles gambiae* saliva protein were included. Unfortunately, Ags specific for *P. ovale* or *P. knowlesi* were not available and could therefore not be included.

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SEROLOGICAL TOOLS FOR MALARIA: WHAT ARE THE POTENTIALS?

THIS OPINION REVIEW WILL BE SUBMITTED AS:

Kerkhof Karen[1,2§], Coosemans Marc[1,2§]. Serological tools for malaria: what are the potentials?

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Serology: an innovation method in low endemic settings?

A better understanding of the risk factors associated with malaria infection in low endemic countries can support programs that aim to reduce the malaria burden to eliminate malaria [1]. In these settings, traditional metrics (e.g. Entomological Inoculation Rate (EIR), parasite rate (PR)) appear to be less suitable to quantify the malaria infection. In low endemic settings, where parasitaemia and/or vector density become lower, measuring EIR is difficult as it is often measured between 1-5 infective bites per year [3–5]. Alternatively, PR has the advantage of being measured by PCR detecting lower levels of parasitaemia (up to 0.2 parasites/µl blood [6]), as compared to microscopy and Rapid Diagnostic Tests (approximately 4-100 parasites/µl blood [7, 8]). However, these techniques are complex, time consuming, and require special training with high equipment and consumable costs [9]. These limitations reduce their potential in low endemic settings [1]. As such, serology is proposed as a reliable and sensitive method in low endemic areas to assess malaria epidemiology [10].

Serology focusses on the detection of antibodies (Abs) against *Plasmodium* antigens (Ags) in human samples [11]. It is designed with the rationale (i) to detect past and recent infection and (ii) to assess the level of protective immunity against clinical symptoms [12]. Therefore, it is suggested that Abs offer a wealth of information about the exposure to malaria parasites. In contrast to PR approaches, in low transmission settings, serological measures may give more precise estimates with the use of smaller sample sizes. This allow a more sensitive and specific measure of exposure at population level [13].

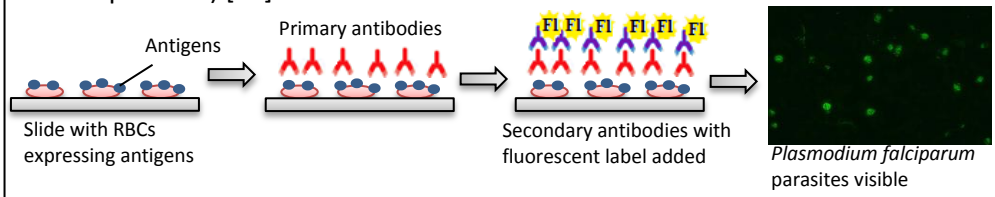
The features of malaria serology

Malaria serology plays a major contribution in monitoring and evaluating malaria exposure [7] over multiple timescales. It is mainly used in areas where the probability of exposure and current infection is low. In these settings, serology could be a valuable means to identify an at-risk population [14] by obtaining information about current or past parasitic contact [1]. Thus, using serology to assess endemicity and to identify focal areas of transmission will support malaria control as well as elimination programmes [3]. In contrast, serology does not support the diagnosis of acute malaria [7], although serology may be useful for those who focus on the protective immunity against clinical malaria [15]. Defining a protective threshold for malaria with serology is challenging [16] because the degree of immunological protection against malaria is not controlling the infection as such, but rather is against the clinical symptoms once it turns parasitemic [17]. Most closely related research focusing towards protective immunity is based on defining the disease incidence according to age [18], however to

date, no concrete results have been perceived yet. This is beyond the means and the scope of this review. Therefore, the main goal is to highlight the epidemiological purposes in context of malaria control and elimination programmes.

Box I. Indirect ImmunoFluorescence Antibody test (IFAT)

The measurements of malaria Abs date back to the 1960s and were mainly used in epidemiological studies. The standard technique was the indirect Immuno Fluorescence Antibody Test (IFAT) [19–22]. Due to its sensitivity and simplicity to perform, it was for a long time the only approved and reliable test for malaria Ab detection in epidemiological studies [7, 68]. The principles of the IFAT are based on thin and thick parasite blood smears in which whole parasites constitute the Ag [24]. The idea was that by making use of the entire parasite, no extraction methods were required as in case of the former techniques (e.g. precipitation, agglutination, complement fixation, haemagglutination and immune-electrophoresis) [68].



Briefly, the IFAT is a manual straightforward method [13] with a high sensitivity [68]. However, due to the need of a fluorescence microscope, trained personnel is needed for reading and interpreting the outcomes, especially in serum samples with low Ab-levels. This makes the IFAT time consuming and difficult to automate [7, 55]. Furthermore, by utilizing red blood cells only native blood stage Ags can be detected, and the IFAT is mainly validated for *P. falciparum* [54]. Note that up to this moment the IFAT is still regarded as the golden standard for malaria

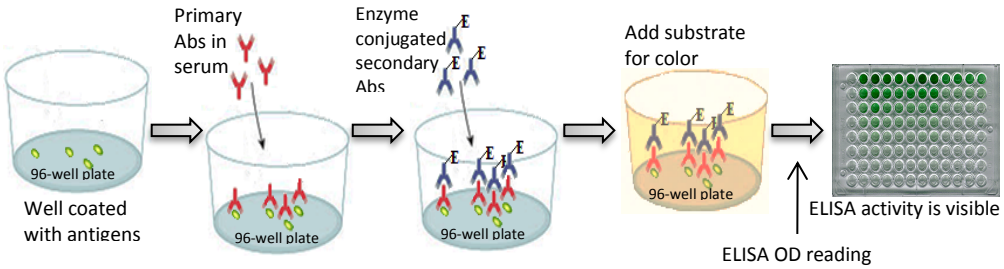
Malaria serological tools

Through the years, evaluating malaria transmission by means of the detection of Abs against *Plasmodium* Ags has been carried out in various ways. These measurements of malaria Abs date back to the 1960s and were mainly used in epidemiological studies. Standard techniques were the indirect Immuno Fluorescence Antibody Test (IFAT) [19–22] and the Enzyme Linked Immunosorbent Assay (ELISA) [7, 22, 23]. The IFAT (Box I.) is a technique based on thin and thick blood smears to detect Abs against the *Plasmodium* parasite in its native form [24]. However, this technique was not capable to distinguish the different life-cycle specific. Ags ELISA (Box II.) was introduced to

overcome this obstacle and detect Abs against a range of life-cycle specific *Plasmodium* Ags or parasite extracts. Even so, ELISA can only detect one Ag per well (96-well plate) per time [25], which is a major drawback when performing large-scale serological studies. As a response to the increasing demand for approaches that have a higher throughput, a novel tool was developed in 2012: the multiplex bead-based immunoassay (MPA) (Box III.) [26]. This technique is comparable to a standard ELISA, yet it allows simultaneous measurement of a variety of Abs against *Plasmodium* Ags [13].

Box II. Enzyme Linked ImmunoSorbent Assay (ELISA)

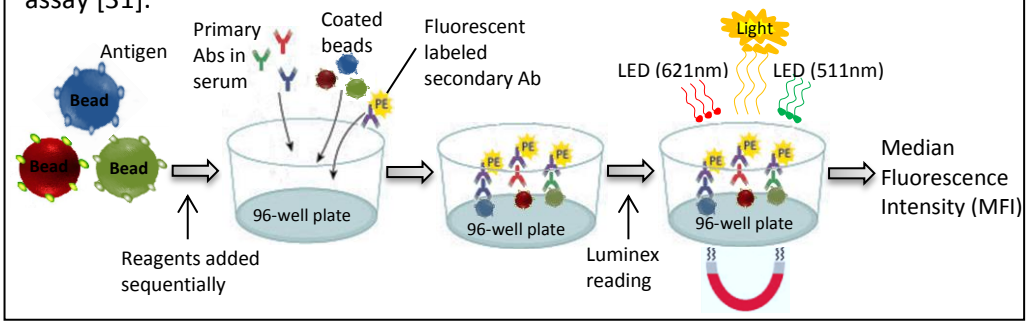
The indirect ELISA starts when Ags are coated to the surface of a well in a 96-well plate. Thereafter, blocking buffer is added to block the remaining protein-binding sites on the surface of the wells. Then, blood serum that contains the primary Ab is added into the well, and thereafter a conjugated detection Ab. This detection Ab is a colorant or an enzyme used for the detection of the reaction. In contrast to the IFAT, a single Ag is now used instead of the whole parasite who produces Ags that can cover the entire life-cycle at once [25].



Both IFAT and ELISA are sensitive, but the ELISA has a higher specificity [7]. Another difference is that IFAT focusses on the native Ags, whereas ELISA can combine native and recombinant proteins [55]. Unfortunately, with the advent of the ELISA the labour intensity did not decrease. Furthermore, the ELISA also requires large amounts of serum [58]. In order to improve these techniques, it was essential to focus on a technique that requires less serum, that is capable of detecting more than one Ab-response per time, and has a higher throughput [58]. As such, the multiplex bead-based immunoassay was developed.

Box III. Multiplex Bead-Based Immunoassay (MPA)

The first MPA (dual-laser Luminex-100 system) was released on the market in the late 1990s [69]. To use a MPA, different microspheres/bead sets are coupled with the desired Ags. These beads are internally dyed with different red and infra-red fluorochrome intensities (maximum of 100 various colour codes based microspheres). Due to this, these beads can be measured simultaneously and allow for detection of Ab-responses against a maximum of 100 different Ags per well [70] in very small volumes [69]. This test is extremely cost- and time effective, requires only a minimum amount of serum, and several Ags can be tested at once. Additionally, its sensitivity and specificity is comparable, as both techniques are carried out directly on the Ags, with (or even better than) an ELISA assay [31].



It is of great importance to obtain good knowledge concerning the biological activity and Ab-persistence of the used serological markers, since it could greatly benefit the outcomes in different transmission settings when used in the available serological systems. Therefore, the available serological markers and corresponding Ab-responses are briefly discussed in Figure 1.

Limitations and possibilities of these techniques

The first studies concerning acquired immunity of malaria date back as early as the year 1900 [19, 27–33]. Stephens & Christopher's [28], Koch [29–32] and Plehn [33] performed the first serological studies on people from indigenous populations in Cameroon. They were thereby able to prove that adults in these populations exhibited a form of immunity after repeatedly being infected [19, 34]. Consequently, several theories emerged to explain the acquired immunity by making use of the standard bacteriological serological techniques (e.g. precipitation, agglutination and complement fixation). Unfortunately, each of these approaches led to below par results. As such, studies to find newer serological tests were essential [19].

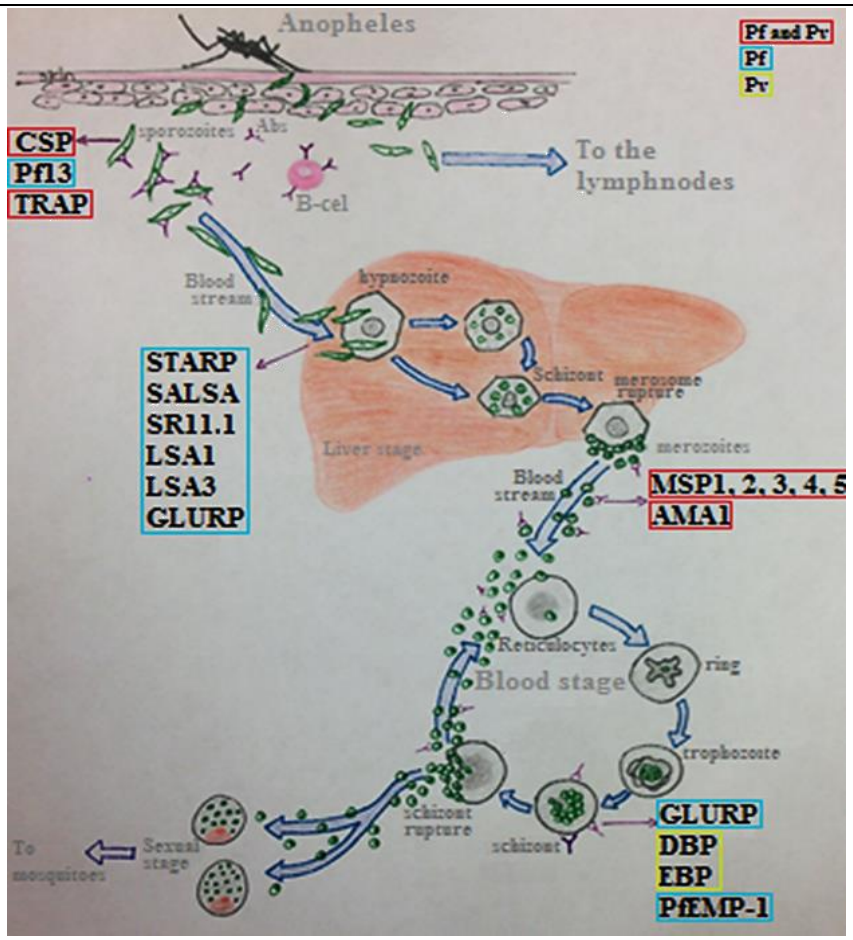


Figure 1: Most commonly used Ags in serological assays and corresponding their life-cycle stages.

The infectious forms of the malaria parasite transmitted by mosquitoes are the *Plasmodium* sporozoites that initiate the immune response in the human host [35]**. An average of 10-100 sporozoites are known to remain in the skin of the host,** [36, 37] **leaving a trail of circum-sporozoite-proteins (CSP)** [36]**. They then proceed to the liver to traverse Kupffer cells and hepatocytes, after which *Plasmodium* develops in the nucleus of the hepatocytes, by letting the sporozoites undergo nuclear replication** [37]**. At this phase, solely the liver-stage Ags are expressed at the surface of the infected hepatocytes. Of these Ags, the most commonly known are the liver-stage antigen 1 (LSA1) and liver-stage antigen 3 (LSA3)** [38]**. After nuclear replication, mature liver eventually develop, which release merozoites into the bloodstream** [36]**. These merozoites are ovoid cells containing apically located secretory organelles. Merozoites remain in the bloodstream for a short time and produce proteins needed for invading new erythrocytes. It is in this short timespan that Abs are in direct contact with the merozoites. As such, merozoite proteins have long been considered to constitute the best target for serological assays and vaccine development** [39]**. Most commonly known and used merozoite proteins are the merozoite surface protein (MSP1), apical membrane antigen (AMA1)** [40]**, as well as the less applied Duffy binding protein (DBP** [41]**) and erythrocyte binding protein (EBP** [42]**). Other leading candidates in serological assays are Glutamate Rich proteins (GLURP) that produce Ab-responses related to the protection against malaria in exposed populations. This is an antigen that is expressed at the surface of the host cell, in all stages of the *Plasmodium* life cycle in the human host** [43, 44]**.**

(This figure is a sketch that will be improved by a professional illustrator before submission)

Subsequently, newer immunological tests such as haemagglutination, immune-electrophoresis and fluorescent antibody arose. These tests were most often performed on serum from rats infected with either *P. berghei* or *P. vinckei* [45], and over time on *P. falciparum* and *P. vivax* as well. Therefore, soluble Ag attached to aldehyde-fixed type "O" erythrocytes were used to evaluate the serum samples [46]. Researches interpret the process of agglutination that depends on the forces that drive the erythrocytes together or apart [19, 45, 47]. In addition, the immune-electrophoretic techniques, an agar electrophoretic method used to detect Ags and Abs in serum, proved to be useful in serology of malaria [19]. This was already an advantage pertaining to the previous used methods, as it is a faster and simpler one-step procedure. It is based on an electro-endosmosis process in which an anodal Ag forms a complex with a cathodal Ab. When this process happens a precipitate will be visible and detectable [19, 48]. Nonetheless, it was the fluorescent Ab test that represented a major break-through in the field of serology. This is because IFAT proved it purposes in several ways, namely; (1) in defining recent malaria transmission, (2) in the framework of control and intervention studies, and (3) in epidemiological studies.

In a first instance, IFAT was used to demonstrate whether a foci in a low transmission area was still active [22]. To prove the active foci on the basis of malaria transmission and Ab-levels, these studies mainly focused on populations in the age between 2 and 50 years old. The estimation of the malaria endemicity was based on a variety of factors [19, 49]: (1) to perceive differences in seroprevalence between different study areas, (2) to demonstrate an increase according to age that reflects the exposure over time, (3) to observe an increase in Ab-levels together with age between different study areas, and (4) the entomological inoculation rates and IFAT-titers must be strongly related [49].

Besides using IFAT to estimate recent and past malaria transmission, it was also useful in the scope of control and intervention trials [50, 51]. Therefore, several serological techniques (mainly IFAT and haemagglutination Ab) were used to study the Ab-responses in populations (infants, children and adults) over time, during and after the application of control measures [50]. The study design was mainly based in highly endemic areas of West Africa where *P. falciparum* dominates. These serological tests included IgG and IgM Abs, to observe relationship with the parasitological data. With IFAT it was possible to observe Ab-levels that increase rapidly with age and reached a plateau at a certain age. This is a reflection of the development of an active immune response [51]. These serological results were used to observe a reduction in malarial

antigenic stimulation based on the control measures applied. Changes observed in the serological results of populations that received protection depends on both the effectiveness of the protection and the prior history of malaria exposure [51].

A third approach in which IFAT can be used is epidemiological studies [52]. IFAT showed good correlations with the parasitological data, in which differences were obtained when a plateau was reached in function of age (Figure 1A). A sudden increase of Ab-responses could be observed at the beginning of the transmission season (Figure 2B). The effect of the increase in transmission is much more intense in children, however, IFAT allows also to detect the onset of transmission in older age groups as well.

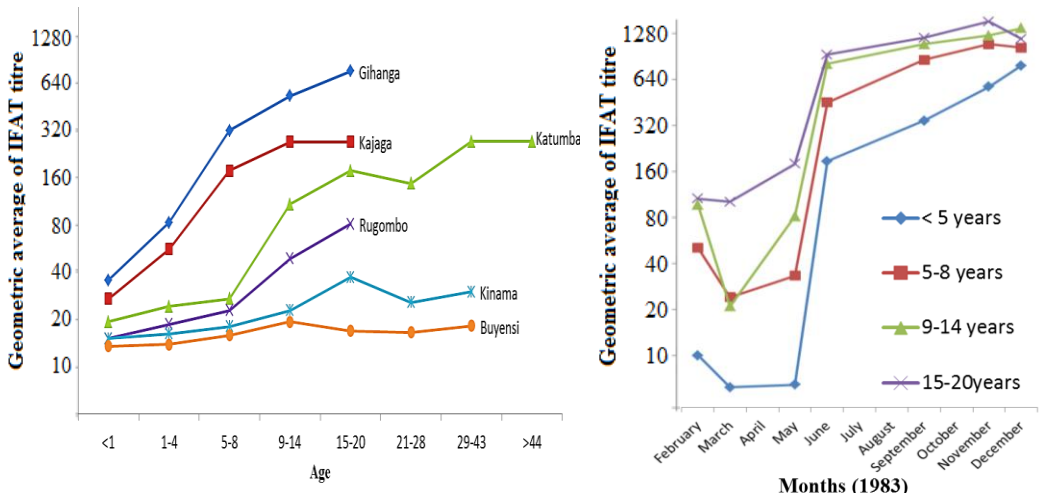


Figure 2: (A) Geometric averages of titres obtained with the indirect immunofluorescence test (IFAT) according to age and locality. Source cited from Coosemans *et al.* [20]. (B) Evolution of Abs-levels perceived with the indirect immunofluorescence test (IFAT). Source cited from Coosemans *et al.* [21]. Both sources were approved for usage by Coosemans *et al.* [20, 21].

Through the years IFAT proved to be a good serological measurement, although some limitations have been observed. It is restrained by reliance on cultured parasites which mainly focused on *P. falciparum*, expensive fluorescence microscopes, the need for high skilled technicians, and the subjective nature of slide reading [53]. Another drawback of IFAT is the significant cross-reactivity observed between the different *Plasmodium* species [54, 55]. Therefore, ELISA became the new potential measurement of antimalarial Abs in epidemiology for its utility of screening a combination of specific Abs [53]. At first, ELISA was used with IFAT as a reference, but

when ELISA presented a low sensitivity in the context of plasmodial infections it raised consternations. However, in ELISA it was possible to utilise recombinant proteins and peptides against multiple *Plasmodium* species. Thus, ELISA showed a superior performance over IFAT [11], because IFAT concentrates on Ags derived solely from *P. falciparum*. A major drawback is that ELISA is only capable of screening Ab-responses against a single Ag at a time, and that cross-reactivity can be observed between different *Plasmodium* species in both IFAT and ELISA. The cross-reactivity can affect the specificity of the test in areas were more *Plasmodium* species co-occur [56], but this effect should have reduced when using recombinant proteins. Another drawback is that there is no 'gold standard' for ELISA. Logistically IFAT should be the 'gold standard', yet, this is only possible when IFAT is used to detect Abs against *P. falciparum* Ags [11, 55]. Overall, better efficiency and reliability in the context of malaria transmission and epidemiological studies have been observed with ELISA [55].

One step forward in the serology was the innovation with the multiplex bead-based immunoassay (MPA). This serological technique made it possible to incorporate multiple Ags. The use of multiple specific Ags simultaneously is an advantage, because no single marker can serve as the sole marker for malaria exposure [13], and no different screenings are needed. Thereafter, several studies focused on estimating the half-lives of different Abs, presented in Table 1, in which a wide range of different half-lives was found. A remarkable point is that MPA shows a higher reproducibility compared to IFAT and ELISA, and is furthermore easier to implement, better to automate and does not rely on a subjective reading as is case for the IFAT [58–62].

Table 1: Representing the antibody half-lives of the most commonly used serological markers [57]**.**

Antibody half-lives			
< 7.5 months	**8.5 – 12 months**	**13 -19 months**	**>2 years**
GLURP	STARP.R	SALIV.1	PvEBP
SR11.1	Pf13	LSA1	CSP (vivax)
LSA3	CSP (falciparum)	PvAMA1	PvDBP
Pf.MSP1.19	SALSA2	Pv.MSP1.19	
	SALIV.2		

Even though the benefits of this technique are well known, purchasing a Luminex device remains cost prohibitive for many places. There is need for an extensive training on how to use the technique and interpret the data outcome. Getting deeper into the data interpretation, there is a need for statistical modelling taking into account all Ags simultaneously, while knowing that not every individual responds

similarly. Presently, the results of the MPA and ELISA are still statistically analysed Ag per Ag, mainly by means of a reversible catalytic conversion model (threshold model) [63–65, 53]. An Ab-response varies by infection, so to be able to detect recent infection, this should be linked to IFAT, which takes into account all blood stage Ags and perceives one single outcome. This could be even further improved, when the threshold model is replaced by a model that is based on the Ab intensity data (raw continuous data). The threshold model is a simplification of a complex immunological process [53], whereas the use of Ab intensity data can provide more quantitative changes [53, 66]. These drawbacks are critical for making serological assays acceptable to control programmes, and should be further investigated.

Traditionally the focus was on the conventional Ags (AMA1 and MSP1) measured separately. This may limit future progress, as it is know that these Ab-responses have a long half-life. This prevents the possibility to distinguish very recent exposure from more distant exposure. It is suggested that serology, and in particular MPA, is a unique attribute to provide auspicious information on transmission patterns, once a serological marker for recent exposure in low transmission settings is validated [67]. Once the right combination of serological markers is established, it will allow for determining recent as well as past exposure exists. This technique will be a major step forward in estimating malaria transmission, in control and intervention studies, and for seroepidemiological purposes.

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IMPLEMENTATION AND APPLICATION OF A MULTIPLEX ASSAY TO DETECT MALARIA SPECIFIC ANTIBODIES:

A PROMISING TOOL FOR ASSESSING MALARIA TRANSMISSION IN SOUTHEAST ASIAN PRE-ELIMINATION AREAS.

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Abstract

Background: Epidemiological surveillance is a key activity in malaria control and elimination in low transmission and pre-elimination settings. Hence, sensitive tools for estimating malaria force of infection are crucial. Serological markers might provide additional information in estimating force of infection in low-endemic areas along with classical parasite detection methods. Serological markers can be used to estimate recent, past or present malaria exposure, depending on the used markers and their half-life.

Methods: An assay based on fourteen *Plasmodium* specific peptides, one peptide specific for *Anopheles gambiae* saliva protein and five *Plasmodium* specific recombinant proteins was developed for the MAGPIX system, assessed for its performance, and applied on blood spots from 2,000 individuals collected in the Ratanakiri province, Cambodia.

Results: A significant correlation for the use of 1,000 and 2,000 beads/antigen/well as well as for the monoplex *vs.* multiplex assay was observed for all antigens ($p<0.05$). For the majority of antigens, antigen coupled beads were stable for at least two months. The assay was very reproducible with limited intercoupling, interplate and intraplate variability (mean RSD < 15%). Estimating seroconversion and seroreversion per antigen using reversible catalytic models and models allowing two seroconversion rates showed higher seroconversion rates in adults.

Conclusion: The multiplex bead-based immunoassay was successfully implemented and analysis of field blood samples shows that changes detected in force of malaria infection vary according to the serological markers used. Multivariate analysis of the antibody responses and insights into the half-life of antibodies are crucial for improving the interpretation of these results and for identifying the most useful serological markers of past and recent malaria infection.

Keywords: Malaria, serological markers, multiplex immunoassay, Cambodia

1. Background

Globally an estimated 3.4 billion people in 107 malaria-endemic countries are at risk of malaria, of which 1.2 billion at high risk [1] live mostly in the African region (47%) and the Southeast Asian region (37%). To control and eliminate malaria, WHO recommends a multi-pronged strategy, which includes vector control interventions, preventive therapy, diagnostic testing, treatment with quality-assured artemisinin-based combination therapy (ACT) as well as strong epidemiological surveillance [1]. Through upscaling of several elements of this strategy, many countries are on the verge of reaching pre-elimination. The low transmission rates in these areas pose considerable challenges for epidemiological surveillance [2], hindering the evaluation of new (vector) control tools necessary to reach elimination [1].

Detection of malaria-infected persons by microscopy, rapid diagnostic tests (RDTs) and even PCR lacks sensitivity because of low numbers of positive samples [3], representing a formidable logistical challenge due to the very big sample sizes required in surveillance and evaluation surveys. While parasite-prevalence (measured by microscopy, RDTs or PCR) provides a snapshot of the exposure to malaria, the use of serological markers can provide a picture of the malaria transmission over a prolonged period [3,4]. Serology is based on the detection of antibodies (Abs) against antigens (Ags) of malaria parasites, which offers an advantage as anti-*Plasmodium* Abs can persist for months after infection. Therefore, these Abs have been suggested as indicators of malaria transmission [5-8], and are believed to be a better approach to determine past, recent and present malaria exposure [9]. Previous studies performed in low transmission settings, such as Cambodia, also suggest that serological assays are promising for indicating malaria transmission [3, 10–12].

Since the 1960s, serological markers detected by indirect immunofluorescence antibody tests (IFAT) were used to assess malaria transmission intensity and reductions in transmission [13]. This has proven to be a reliable and useful serological test for malaria in epidemiological surveys [5,6,14]. However, variation in source of Ags and the subjectivity of IFAT has led to this method falling out of favour [4]. Standardized tests based on recombinant Ags used in an enzyme-linked immunosorbent assay (ELISA) were therefore developed [4,7-9]. However, an ELISA can only assess one marker at a time, making it labour intensive and time consuming when interested in multiple Ab responses. In the context of malaria elimination it will become essential to take into account individual variations in Ab responses, the occurrence of multiple malaria parasites [15], as well as to increase the probability of measuring changes in Ab responses by combining different markers. Recently, several

multiplex assays that were testing for different serological markers in the same blood sample, were developed by different research teams based on the Luminex technology [15-18].

In this context, the general objective of this study was to implement an existing assay based on the Luminex technology for detection of Abs against malaria parasites in blood samples from Ratanakiri Province, Cambodia. This is the first and most extensive multiplex assay in malaria serology executed in the Southeast Asian region, including 20 Ags (recombinant proteins and peptides) directed against different specific malaria parasites. Furthermore, this study includes a detailed analysis on the stability of coated beads over time and the reproducibility of the beads coupling and immunoassay.

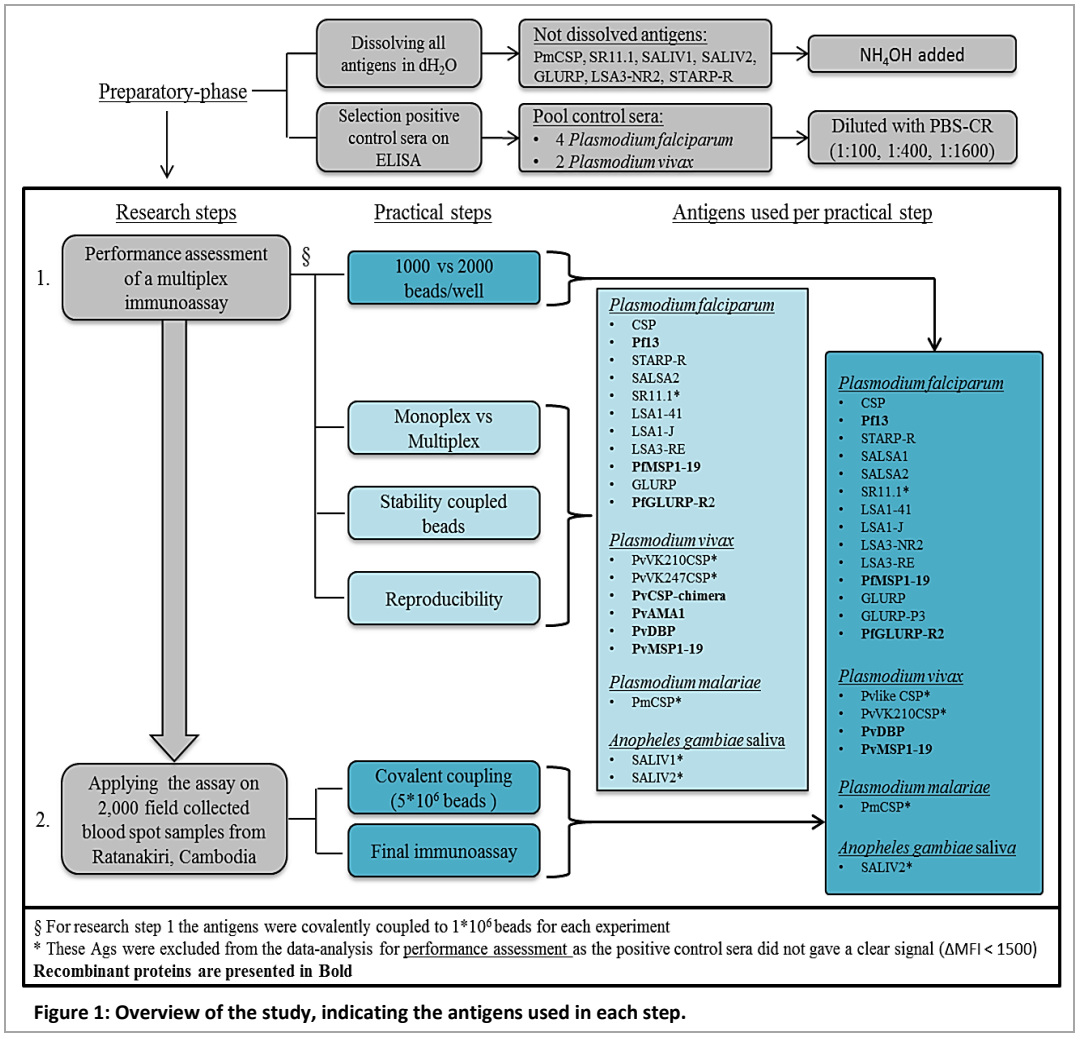

Figure 1: Overview of the study, indicating the antigens used in each step.

2. Methods

2.1 Samples

A positive control for the assay was prepared by pooling sera from four *Plasmodium falciparum*- and two *Plasmodium vivax*-infected patients from Ratanakiri Province in Cambodia. Dilutions of this positive control pool were prepared at 1:100, 1:400 and 1:1,600 in PBS-CR (phosphate buffered saline, Charles River Laboratories Inc, MA, USA). These dilutions were used to assess the performance of the assay and as positive control samples in the immunoassay applied on field blood samples. The latter were collected in Ratanakiri Province, Cambodia, on filter papers through finger prick, during April-May 2012 (baseline survey of the MalaResT project, NCT01663831, which aims to evaluate the use of topical repellents, in addition to long-lasting insecticidal nets, on malaria prevalence and incidence [19, 20]). Out of 5,392 blood spot samples collected, 2,000 were randomly chosen for the immunoassay and after quality control, 1,931 samples were used for data analysis. Blood spot filter papers were prepared by punching two discs of 4-mm diameter, and eluted overnight in 160 µL of PBS-TBN (dilution 1:40, PBS-1% BSA-0.15% Tween, pH 7.4, Sigma-Aldrich). Just before use in the immunoassay, the eluted samples were further diluted to 1:200 in PBS-CR.

2.2 Antigens

Selection of peptides specific for *P. falciparum* representing different life stages of the parasite was based on the work of Ambrosino *et al*. [5]. Additionally, peptides specific for *Anopheles gambiae* saliva protein [5], *P. vivax* and *Plasmodium malariae* were included in the assay, as well as specific recombinant proteins for *P. falciparum* and *P. vivax* (Table 1). All peptides were chemically synthesized with an added N-terminal cysteine residue and bovine serum albumin (BSA) (Table 1) [5] by GeneCust Europe (Dudelange, Luxembourg). The recombinant proteins were synthesized as described in Table 1. This study consisted of two phases (performance assessment of the assay, and application to field samples; Figure 1). For practical reasons, some steps carried out during the performance assessment used a slightly different Ag set (Figure 1).

2.3 Covalent coupling of antigens to the beads/microspheres

Covalent coupling of paramagnetic beads (MagPlex microspheres, Luminex Corp, Austin, TX, USA) was carried out as described by Ambrosino *et al.* [5] and the Luminex Corp [21,22]. Each Ag was coupled at a concentration of 4 µg Ag/10^6 beads to 1x10^6 beads/beadset for performance assessment (Figure 1). When applying the assay on blood samples from Ratanakiri, all Ags were coupled twice to 5x10^6 beads/beadset,

and mixed for homogenous coupling. BSA (Sigma-Aldrich, St Louis, USA) was coupled to an additional set of beads to serve as a background control [5,23].

Table 1: Overview of the antigens (peptides and recombinant proteins) used in this study.

Antigens	Sequence (N-terminal to C-terminal)	g/mol	Life-cycle stages	*Plasmodium species*	Peptide or recombinant protein	Ref
CSP	NANPNANPNANPNANPNVDP NVDPC	2557.67	Sporozoite	*P. falciparum*	Peptide	[5]
Pf13	C-terminal His-tag produced in *E.coli*		Sporozoite	*P. falciparum*	Recombinant proteins	[45, 46]
STARP.R	STDNNNTKTISTDNNNTKTIC	2299.42	Sporozoite & liver stage	*P. falciparum*	Peptide	[5, 47]
SALSA1	SAEKKDEKEASEQGEESHKKENS QESAC	3123.24	Sporozoite & liver stage	*P. falciparum*	Peptide	[5, 47]
SALSA2	NGKDDVKEEKKTNEKKDDGKTD KVQEKVLEKSPKC	4019.52	Sporozoite & liver stage	*P. falciparum*	Peptide	[5, 47]
SR11.1	EEVVEELIEEVIPEELVLC	2213.5	Sporozoite & liver stage	*P. falciparum*	Peptide	[5, 47]
LSA1.41	LAKEKLQEQQSDLEQERLAKEKL QEQQSDLEQERLAKEKEKLQC	5297.97	Liver stage	*P. falciparum*	Peptide	[5, 6, 48]
LSA1.J	ERRAKEKLQEQQSDLEQRKADT KKC	3046.43	Liver stage	*P. falciparum*	Peptide	[5, 47, 48]
LSA3.NR2	VLEESQVNDDIFNSLVKSVQQEQ QHNVC	3230.53	Liver stage	*P. falciparum*	Peptide	[5, 47]
LSA3.RE	VESVAPSVEESVAPSVEESVAEN VEESVC	2991.2	Liver stage	*P. falciparum*	Peptide	[5, 47]
Pf.MSP1.19	Glutathione S-transferase (GST) fusion protein. C-terminal expressed in *E.coli*		Merozoite	*P. falciparum*		[4, 24]
GLURP	EDKNEKGQHEIVEVEEILC	2241.47	Trophozoite	*P. falciparum*	Peptide	[5, 47, 49]
GLURP.P3	EPLEPFPTQIHKDYKC	1945.23	Trophozoite	*P. falciparum*	Peptide	[5, 47, 50]
Pf.GLURP.R2	C-terminal produced in *E.coli*		Trophozoite	*P. falciparum*		[49]
Pvlike.CSP	APGANQEGGAAAPGANQEGGA AAPGANQEGGAAC	2892.99	Sporozoite	*P. vivax*	Peptide	[47]
PvVK210.CSP	DGQPAGDRAAGQPAGDRADG QPAGDRADGQPAGC	3206.3	Sporozoite	*P. vivax*	Peptide	[47, 51, 52]
PvVK247.CSP	ANGAGNQPGANGAGNQPGAN GAGNQPGANGAGNC	2905.95	Sporozoite	*P. vivax*	Peptide	[47, 51, 52]
PvCSP chimera	Soluble His-tag protein expressed in wheat-germ cell free expression system		Sporozoite	*P. vivax*	Recombinant proteins	[51, 52]
PvAMA1			Merozoite	*P. vivax*	Recombinant proteins	[53, 54]
PvDBP			Merozoite	*P. vivax*	Recombinant proteins	[55]
Pv.MSP1.19	C-terminal produced in the baculovirus expression system		Merozoite	*P. vivax*		[4, 16, 24]
PmCSP	GNAAGNAAGNDAGNAAGNAA GNAAGNAAGNAAGNAAC	2358.37	Sprozoite	*P. malariae*	Peptide	[47]
SALIV1	EKVWVDRDNVYCGHLDCTRVA TFC	2830.22	Salivary gland proteins	*An. gambiae*	Peptide	[5, 56]
SALIV2	ATFKGERFCTLCDTRHFCECKETR EPLC	3324.84	Salivary gland proteins	*An. gambiae*	Peptide	[5, 56]

Ags are organized according to the *Plasmodium* species and the life-cycle stages in the human host

2.4 Bead-based immunoassay

The immunoassay was carried out as described previously [5,17,22,24,25], with minor adjustments. A microsphere working mixture was prepared in PBS-CR, with a concentration of 1,000 beads/Ag/well (except in the experiment to assess the difference between a concentration of 2,000 and 1,000 beads/Ag/well). For the monoplex assay the microsphere working mixture consisted of only one beadset with a coupled Ag, whereas it consisted of a pool of all coupled beadsets for the multiplex assay. In a 96-well plate 25 µl of the microsphere working mixture (at 40 beads/µl) was added per well and 50 µl of serum sample (1:200 dilution) [24]. Plates were incubated at room temperature in the dark for one hour on a plate shaker (600 rpm). Plates were washed three times, and 100 µl/well of secondary antibody (R-phycoerythrin^{+}-conjugated AffiniPure F(ab')$_2$ fragment of goat anti-human IgG, Jackson Immuno Research Laboratories) at a dilution of 1:500 was added [24]. Plates were incubated for 30 min in the dark at room temperature, and washed three times. Beads were resuspended in 100 µl of 5% PBS-BSA, pH 7.4, and read by the MAGPIX® system. A minimum of 400 beads per spectral address were analysed and results were expressed as the median fluorescence intensity (MFI) [5,25,26].

2.5 Experiments to assess the performance of the multiplex immunoassay

For performance assessment, two bead concentrations of 1,000 and 2,000 beads/Ag/well were compared. The assays performed in monoplex (each Ag separately) and multiplex (all Ags pooled) were compared. Both assays were carried out in triplicate on the positive control pool dilutions (1:100, 1:400, 1:1,600) as previously described [5,25].

Stability of the Ag coupled beads was assessed by performing a multiplex assay directly after coupling the beads and after storage at 4°C during two, three, four, eight, and 16 weeks.

Reproducibility of the beads coupling was tested by coupling all Ags at three different timepoints, followed by an immunoassay on positive control pool dilutions (inter-coupling variability) [17]. Inter- and intraplate reproducibility of the immunoassay was assessed by performing the assay on the positive control pool dilutions on three different plates at different timepoints in one day. This was carried out on three different days (interplate variability) and in each plate the positive control pool dilutions were analysed in six-fold (intraplate variability) [27]. In addition, the inter- and intraplate variability was assessed by looking at the positive control pool samples added to each plate with the field blood samples.

2.6 Analysis of field samples

The immunoassay was applied on 2,000 field blood samples. Therefore, the microsphere working mixture was prepared at a final concentration of 1,000 beads/Ag/well. All field blood spots were analysed in duplicate on separate plates. In each plate, the positive control pool dilutions (1:100, 1:400, 1:1,600), negative control serum (1:200) and blanco (PBS-CR) were analysed in duplicate. In total, 50 plates were analysed and read by the MAGPIX® system.

2.7 Data analysis

All data were incorporated and analysed with R software package version 3.1.0. [28]. Results were corrected for background signal by subtracting the signal obtained with BSA-coupled beads (MFI_{BSA}) to the median value of the Ag-coupled beads (MFI_{Ag}), defined by: $\Delta MFI = MFI_{Ag} - MFI_{BSA}$ [25].

2.8 Data analysis for performance assessment

Ags of which the positive control sera did not give a clear signal (ΔMFI <1,500) were excluded from the data-analysis for performance assessment. Non-parametric Spearman's rank correlation tests were used to analyse the relation between the ΔMFIs for comparing 2,000 *vs.* 1,000 beads/Ag/well, as well as the monoplex *versus* the multiplex assay. Correlations were considered significant at $p < 0.05$. Segmented regression models were used for assessing the stability of the beads over time (R package 'segmented' [28]). First the breakpoint per Ag and per dilution was estimated, followed by choosing the best linear model through ANOVA tests. Ags showing similar breakpoints in time were grouped and the segmented regression model was rerun on the grouped Ags, taking into account the Ags and dilutions as factor. Mean, standard deviation (SD) and relative standard deviation (RSD ($\frac{SD}{Mean} * 100\%$)) expressed as a percentage of the mean were calculated from the ΔMFI for assessing the reproducibility of the assay.

2.9 Quality control of the multiplex assay when applied on field blood samples

To assure the validity of the plates for screening the field blood samples, a quality control was performed on the ΔMFI values of the high positive control pool samples and on the percentage positivity (PP) calculated from the low positive control pool samples. Results were plotted in Levey Jenning Charts (see Additional file 1) and plates with samples that fell out of -2SD and +2SD were rejected and repeated. After quality control, ΔMFI values of each sample and its duplicate were normalized for each Ag

using the value of the high positive control (which was in the linear range of the assay). For this purpose, ΔMFI was converted to PP ($\frac{\Delta MFI\ samples\ (Ag1)}{\Delta MFI\ High\ positive\ control\ (Ag1)}$*100%*) and then the mean PP of the two duplicate samples was calculated. For each sample, the result was rejected when the RSD (relative standard deviation) of two duplicate results exceeded 30%. The results of a total of 1,931 samples were accepted for further analysis.

2.10 Estimation of the force of infection per antigen

To estimate the seroprevalence per Ag, a cut-off value per Ag was generated by fitting a normal mixture model on the transformed data using the natural logarithm of (PP+1) [4]. The mean of the negative distribution +3SD was defined as threshold for seropositivity [4]. These dichotomized serological results were used to fit a simple reversible catalytic conversion model based on maximum likelihood. This model estimates one seroconversion rate (SCR, λ) that represents the force of infection and one seroreversion rate (SRR, ρ) per Ag for all individuals [4,7,9], expressed per person per year. Next, a model estimating two SCRs and one fixed SRR varying across different age groups was fitted. The breakpoint in age was selected using V-fold cross-validation (VFCV) [10]. VFCV randomly partitioned the data into a validation- and training-set. The cross-validation process was repeated five (V) times on the training set and at least once on the validation set. All V results were averaged to one single value (breakpoint). Partitioning the data avoids overfitting [10,29]. Both models were compared through the likelihood ratio test (LRT) at $p < 0.05$ [10,30].

2.11 Ethical clearance

The study protocol was reviewed and approved by the Cambodian National Ethics Committee on Health Research (Approval 265 NECHR), the Institutional Review Board of the Institute of Tropical Medicine Antwerp (Approval IRB/AB/ac/154) and the Ethics Committee of the University of Antwerp (Approval B300201112714). Gatekeepers provided informed written consent for the participation of their village. The survey participant or his/her parents or guardian provided informed written consent for individual participation.

3 Results

No clear ΔMFI signal was found for the positive control sera for Ags SR11.1, PvlikeCSP, PvVK210CSP, PvVK247CSP, PmCSP, SALIV1 and SALIV2 (ΔMFI <1,500). These antigens were therefore excluded from further analysis in the performance assessment (F 1).

Performance assessment of the multiplex immunoassay

3.1 Using 1,000 performs as good as 2,000 beads/Ag/well

The ΔMFIs obtained from the assays using 1,000 and 2,000 beads/Ag/well were significantly correlated (Spearman's Rank correlation $p<0.05$; $R^2=0.972$; Figure 2A). For most Ags, a slightly higher ΔMFI was observed when using 1,000 beads/Ag/well as compared to 2,000 beads/Ag/well (Additional file 3), and this for all dilutions of the positive control pool (1:100, 1:400 and 1:1,600).

3.2 Multiplexing beads does not affect the assay results

Similar ΔMFI-signals were obtained in monoplex and multiplex (Additional file 3), with a clear correlation for all Ags between the ΔMFIs obtained by the monoplex and the multiplex assays ($R^2=0.972$; $p<0.05$; Figure 2B).

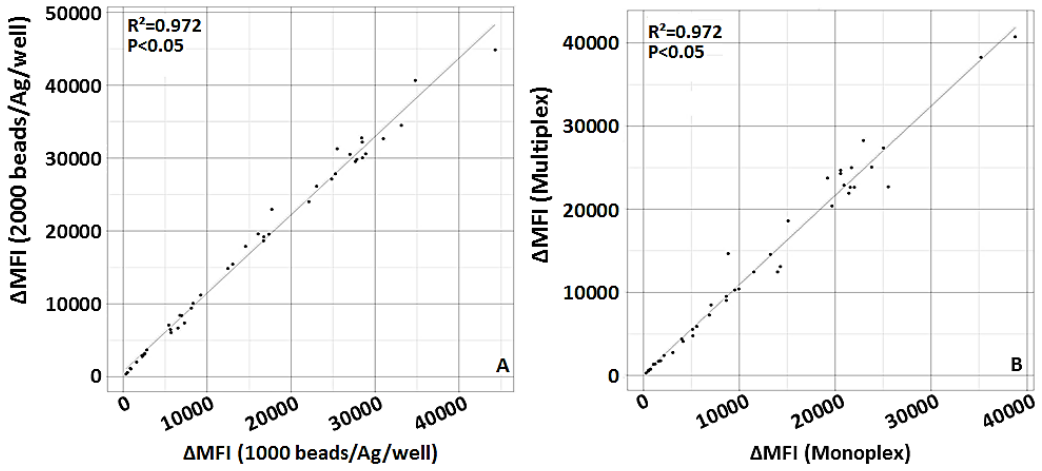

Figure 2: Correlation between the ΔMFI values obtained from analysing the positive control pool with the bead-based assay (A) using 1,000 and 2,000 beads/Ag/well, and (B) in the monoplex and multiplex format.

3.3 Stability of the coupled beads depends on the antigens used

Segmented regression models distinguished three groups of Ags, each with a similar ΔMFI trend over time (Figure 3). For these three groups, different breakpoints (i.e., points in time at which the trend in ΔMFI changes) were observed at three, six and seven weeks after coupling. For the Ags with lower ΔMFI values (CSP, Pf13, STARP-R, SALSA2, PvCSP, PvAMA1, and PvDBP) an earlier break in trend is observed, but with a

low ΔMFI decay over time after the breakpoint (Figure 3 and Additional file 4). In general, for all Ags except for PvCSP, the decay in ΔMFI after eight weeks was less than 10% (Additional file 4). As such, the coupled beads can be used for at least eight weeks after coupling.

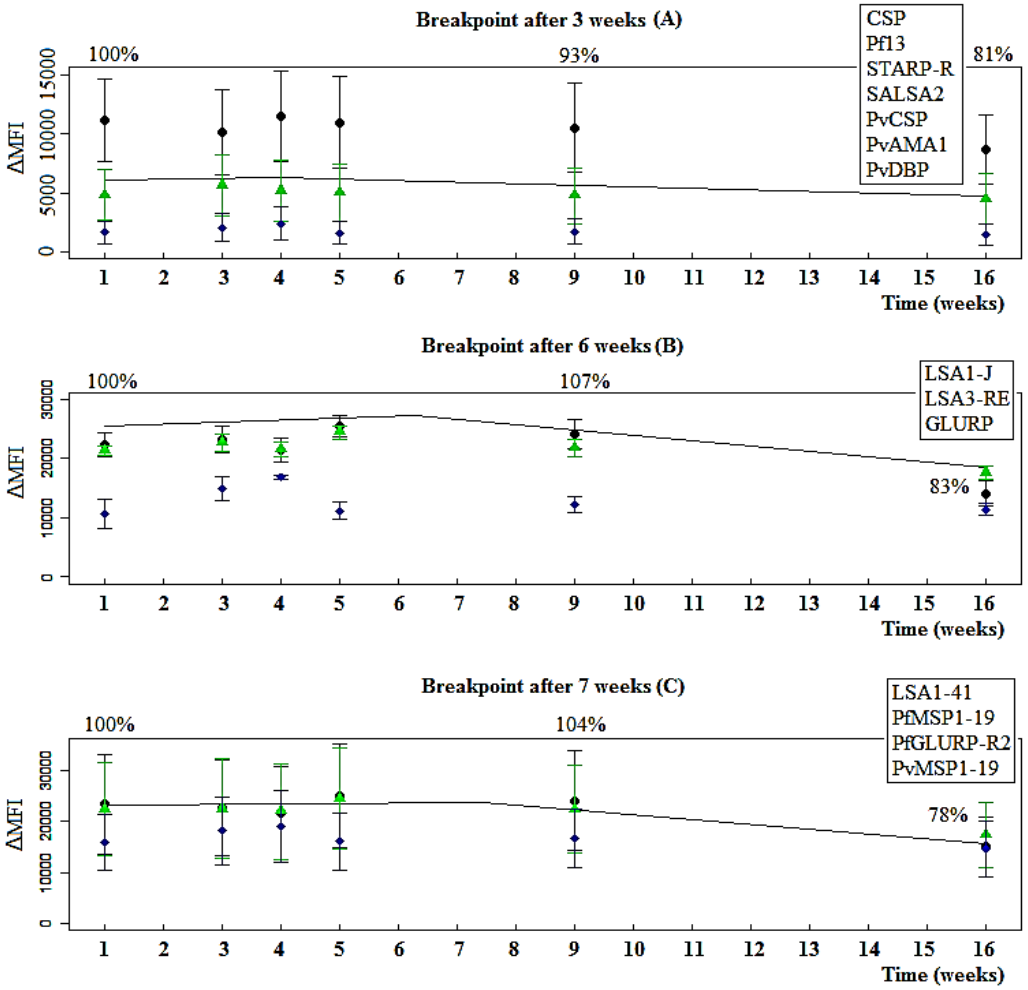

Figure 3: The stability of the coupled beads as determined by segmented regression analysis taking into account Ags and dilutions as factor. Beads were tested at different time points after coupling and the breakpoints in time were estimated by segmented regression analysis. The trend in ΔMFI shows different breakpoints for three groups of antigens, namely (A) three weeks after coupling (CSP, Pf13, STARP-R, SALSA2, PvCSP, PvAMA1 and PvDBP), (B) six weeks after coupling (LSA1-J, LSA3-RE, GLURP) and (C) seven weeks after coupling (LSA1-41, PfMSP1-19, PfGLURP-R2 and PvMSP1-19).

3.4 The immunoassay is reproducible

Intercoupling, intraplate and interplate reproducibility was assessed per Ag by evaluating the RSD values (Figure 4). The ΔMFI results were divided into three

different groups (<5,000; 5,000; ≤15,000; >15,000). Highest RSD values were observed for the lowest ΔMFI values (<5,000). In general, the upper limits of the interquartile ranges were lower than 15% (inter-coupling 11.9% [8.5-15.2%], interplate 7.9% [6.1-12.4%], intraplate 5.7% [3.0-12.7%]) which indicates an acceptable reproducibility [27]. The intraplate and interplate variability was also assessed by analysing the RSD on the ΔMFI of the high, medium and low positive control pool dilutions used as a quality control on the 50 plates with field blood samples (Figure 5). A low variability was observed for the intraplate reproducibility (RSD 2.7% [IQR: 1.2-5.1%]). The interplate variability was higher than experimentally determined during the performance assessment (RSD 18.6% [IQR: 16.5-20.1%], but still acceptable.

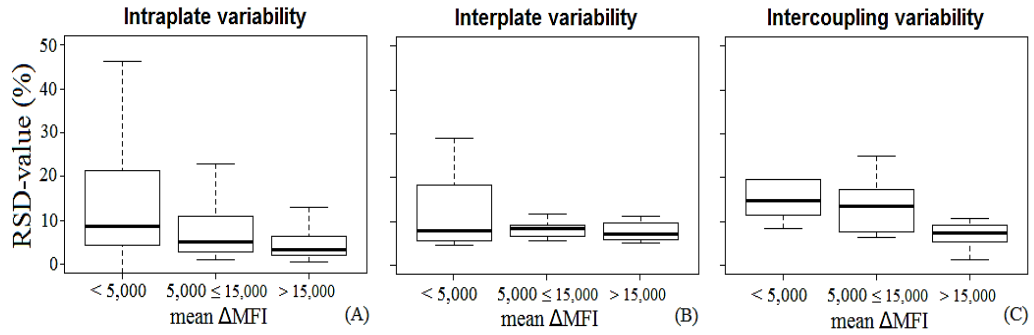

Figure 4: Reproducibility of the bead coupling and immunoassay determined experimentally by analysing the positive control pool. The Relative Standard Deviation (RSD, y-axis) is plotted in relation to the mean ΔMFI values (x-axis) obtained from the assay. The boxplots represent the 75th percentile, median and 25th percentile of the RSD values from the (A) inter-coupling-, (B) intra-plate and (C) inter-plate variability per dilution (1:100, 1:400 and 1:1600). Whiskers represent the maximal and minimal outlier limits.

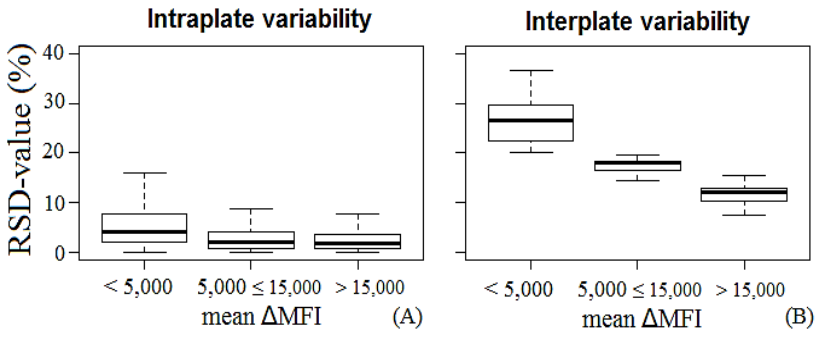

Figure 5: Reproducibility of the immunoassay based on the quality control samples in the immunoassay applied on field collected samples. The Relative Standard Deviation (RSD, y-axis) is plotted in relation to the mean ΔMFI values (x-axis) obtained from the assay. The boxplots represent the 75th percentile, median and 25th percentile of the RSD values from the inter-coupling, intra-plate and inter-plate variability per dilution (1:100, 1:400 and 1:1600). Whiskers represent the maximal and minimal outlier limits.

Application of the assay on blood samples collected in Ratanakiri

3.5 Quality control of the high positive control serum

A total of 2,000 field blood samples were analysed in duplicate in a total of 50 96-well plates. These plates were validated based on the Levey Jenning Charts of the ΔMFI of the high positive controls and the PP of the low positive controls (Additional file 1). The stability of the ΔMFI signal of the positive control pool over the analysis of the plates was confirmed by segmented regression, as no breakpoint could be found for the Ags. Based on the duplicate results of the field blood samples, the results of 1,931 blood samples were validated for further analysis.

3.6 Estimating age-group specific seroconversion rates per antigen

After dichotomizing the serological results for all blood samples based on a cut-off value, age-seroprevalence curves were constructed and reversible catalytic conversion models allowing one and two SCRs were fitted to the data (Figure 6, Additional file 5). The force of malaria infection between age groups was shown for six out of 20 Ags (CSP, SALSA2, LSA3-RE, PfGLURP-R2, PvVK210CSP, and PmCSP), with the time of change obtained through V-fold cross-validated plots (Additional file 6) ranging between ten and 20 years. Higher SCRs were observed for the higher age groups for these Ags. When comparing the SCRs and SRRs between serological markers used (Figure 7), large differences were observed among serological markers used for different life stages of the same parasite, as well as for different parasites. Within the population, highest annual SCRs were modelled for Ags LSA3-RE (0.085 per person per year) and PfMSP1-19 (0.076 per person per year) and lowest annual SCRs for Ags SALSA1, SR11.1, PvlikeCSP, STARP-R, and PvVK210CSP (ranging from 0.007-0.009 per person per year). Highest annual SRRs were modelled for Ags LSA3-NR2 (0.128 per person per year) and SALIV 2 (0.083 per person per year), and lowest for Ags LSA3-RE, PfGLURP-R2, GLURP, SR11.1, PvVK210CSP, and PmCSP (ranging from 0.002-0.005 per person per year).

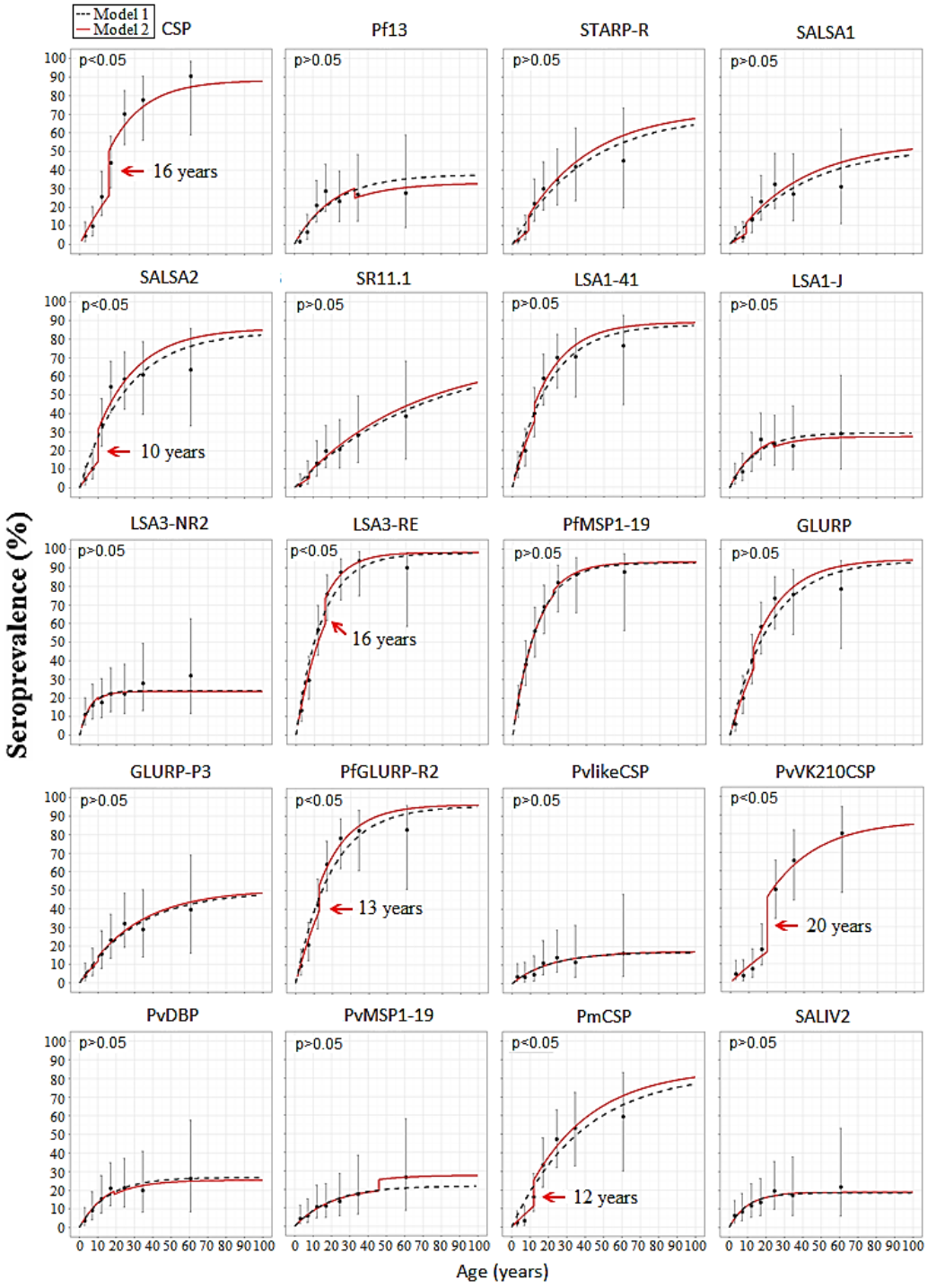

Figure 6: Age-seroprevalence curves for all antigens analysed by the multiplex assay. Reversible catalytic conversion models allowing one (dashed black line, Model 1) and two (red solid line, Model 2) seroconversion rates were fitted to the data. For six Ags the model allowing two SCRs fitted better as compared to one SCR.

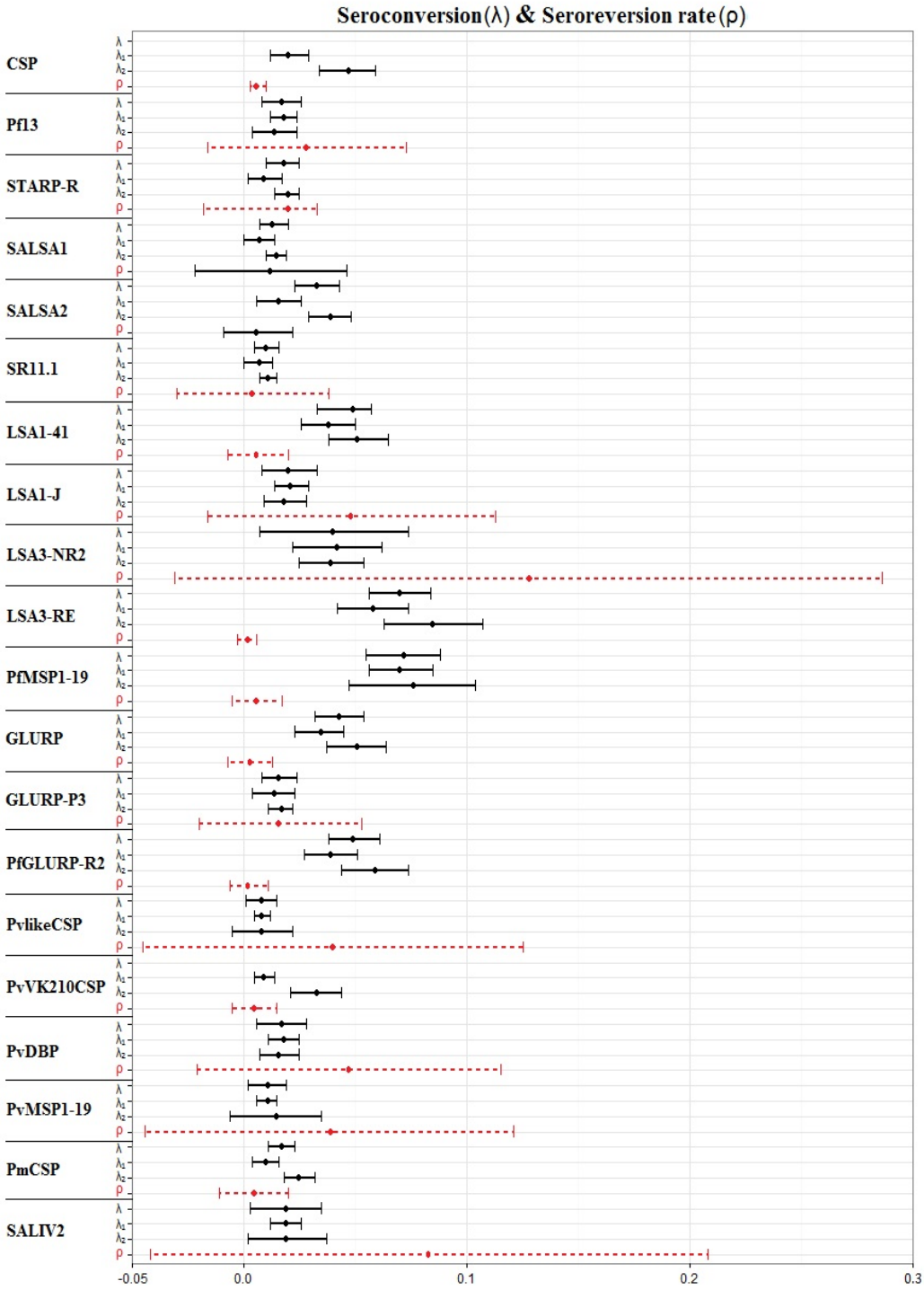

Figure 7: Comparison of Seroconversion and Seroreversion rates between serological markers. The estimated SCRs of reversible catalytic conversion models allowing one (λ) and two (λ1 and λ2) SCRs are plotted with their 95% CI. The SRRs (ρ) are obtained from the model allowing one SCR. Antigens are sorted per parasite and life stage.

4 Discussion

This is the first implemented multiplex assay for malaria serology in Southeast Asia. Moreover, with the use of 20 specific Ags against different *Plasmodium* parasites (Table 1), this assay is the most extensive multiplex assay published on malaria serology, besides Helb *et al.* [32]. In contrast to most studies, the Abs that can be detected are not only directed against one or two different *Plasmodium* parasites [5,10,17,24,25,33], but responses against *P. falciparum*, *P. vivax*, *P. malariae,* and *An. gambiae* are simultaneously detected. This is important in malaria elimination programmes, and especially in Southeast Asia where all four human malaria parasites as well as *Plasmodium knowlesi* are co-occurring [34]. Unfortunately at this stage, Ags specific for *Plasmodium ovale* and *P. knowlesi* were not available and thus not included in the assay. As the final aim of the study was to analyse 2,000 field blood samples, the stability of the coated beads over time and the reproducibility of the assay were first thoroughly assessed and proved to be satisfactory.

Previously it has been shown that multiplex-based serological studies are useful for detecting anti-*Plasmodium* Abs [5,10,15,17,18,24,25] as well as Abs against other pathogens [31,35-38]. Also in the present study, the technique was shown to be reliable and fast in detecting several serological markers at once. All samples were analysed in less than eight weeks of time, and more recently, over 8,000 samples were analysed in duplicate in the same timeframe, showing the possibility to upscale the sample throughput. However, complexity, high investment costs in purchasing a Luminex Analyzer and need for special training, prevent widespread use as compared to ELISA [5,6]. A major restriction of this study is the use of the pool of control serum, as this has to be the same during the entire study, adequate quantities should therefore be prepared and stored in advance. In previous studies, satisfactory evidence has been presented on the validation of bead-based in comparison to ELISA [5,39], and this was, therefore, not further explored in the present study.

Several features were considered to assess the performance of the assay. In accordance to previous observations [17], this study confirmed that a reduction of the beads to 1,000 beads/Ag/well leads to similar results as compared to the amount of beads recommended by the standard protocols for Luminex-based multiplex immunoassays (2,000 or 5,000 beads/Ag/well). A rough cost estimation indicates that the use of 1,000 instead of 2,000 beads/Ag/well reduces the assay costs by one third. Moreover, the results of previous studies [5,15,17] in which monoplex assays resulted in highly comparable ΔMFI values as multiplex assays were confirmed. This refutes the

concern on cross-reactivity between the different Ag-coupled beads or blocking of Ab responses [15].

The obtained results of the performance assessment revealed that most of the coated beads were stable for up to two months, except for Ag PvCSP. This is important as screening approximately 2,000 blood spot samples in duplicate took about seven weeks from the first coupling until the last immunoassay (based on the results 1,143 samples can be analysed per week). This stability is lower compared to previous studies, stating that beads are stable for up to three or seven months [5,33], except for Ag STARP-R which was previously shown to be stable for only one month [5].

Good reproducibility is an important aspect of an assay as it guarantees that results obtained with the assay performed on different plates and at different time points are comparable to each other. In the experimental set-up used in this study, there is no big difference in reproducibility between and within different plates as shown by similar RSDs. This is in line with other studies that showed accurate and repeatable results according to the interplate, intraplate [5,15,27,31], and intercoupling [40] reproducibility of multiplex assays. In this study, the reproducibility was also assessed by looking at the results obtained from the positive control pool dilutions analysed in duplicate on each of the plates used for screening 2,000 blood samples from Ratanakiri Province (total of 50 plates). A much smaller intraplate variability (RSD <3%) was observed compared to the experimental set-up, which was probably due to the fact that samples were only tested in duplicate instead of six times in the experimental set-up. A higher interplate variability was observed, which was still acceptable (RSD <25%) [27], and most probably due to the use of only two positive controls per plate.
In the present assay the use of peptides and recombinant proteins was combined. Fourteen *Plasmodium*-specific peptides (CSP, STARP-R, SALSA1, SALSA2, SR11.1, LSA1-41, LSA1-J, LSA3-NR2, LSA3-RE, GLURP, GLURP-P3, PvlikeCSP, PvVK210CSP, and PmCSP), one peptide specific for the *An. gambiae* saliva protein (SALIV2), and five *Plasmodium*-specific recombinant proteins (Pf13, PfMSP1-19, PfGLURP-R2, PvDBP, and PvMSP1-19) were included in the immunoassay.

After assessment of its performance, the multiplex assay was applied to blood spot samples collected in Ratanakiri Province in Cambodia to detect Ab responses against 20 different Ags. The seroconversion rate (SCR) was then estimated per Ag by constructing age-seroprevalence curves based on a threshold approach. This is currently the most frequently used way of analysing serological data in malaria epidemiology [4,7], but which until the present was not applied to data from multiplex

assays. By fitting models allowing two SCRs [10], age groups exhibiting similar SCRs can be defined. For six out of 20 Ags (CSP, SALSA2, LSA3-RE, PfGLURP-R2, PvVK210CSP, and PmCSP), the models allowing two SCRs fitted better, and a higher SCR was observed in the older age group. This observation corroborates results obtained in previous studies in Cambodia using GLURP and MSP1-19 as serological markers [9]. A lower SCR in the younger age groups may indicate a lower malaria exposure in the age group. This can be attributed to reductions in malaria transmission over time because of intensified malaria control interventions in the last years, or to different risk behaviour linked to the age groups [9]. Moreover, another explanation would be that children might lose their malaria-specific Abs faster [9]. Indeed, previous studies on malaria serology have shown that Ab responses in adults fluctuate to a lesser extent than in children [41,42]. The statistical model fit used to obtain the estimates for the serological parameters was constrained to have two constant (ie age-independent) SCRs and one constant sero-reversion rate (SRR). More elaborate model fitting incorporating age-dependent SCRs and SRRs fell outside the scope of this study.

Until the present very limited information was available on the half-life of the Ab responses to the Ags used and on the individual variation in those responses. This is crucial for interpreting the results obtained with this assay. Serological markers with a long half-life can be used for assessing past exposure, while a short half-life will be useful for assessing recent or even present exposure. The present study has obtained information on the Ab half-life estimated from the SRR as modelled in the reverse catalytic model [43]. Based on this information, fluctuations in Ab half-life were observed per Ag, but all of them seem to have a relatively long half-life (ranging from five to >100 years). Most studies assume a fixed SRR independent of age and transmission rate [30]. A study based on one Ag (PfMSP1-19) [10] compared longitudinal and cross-sectional data (children ages ≤ 11 years), showing a high concordance of SCRs between both study designs, while large discrepancies were seen between the SRRs. A follow-up study based on a selected combination of Ags (10 out of 655) that compared Ab-responses at individual and population level also shows reliable rates of exposure [32]. The present study is based on cross-sectional sampling and the obtained SRRs are probably not reliable. More extensive analysis on the half-life of these Ab responses is planned by using sequentially collected blood samples from the same individuals, as well as by exploring which of the Ab responses can best capture the fluctuations in malaria prevalence and incidence observed over time. SCR estimates based on a single serological marker can give a good indication of the force of infection [4, 7]. However, combining the signal from multiple markers representing different life-stages of the parasite in an individual could help reducing individually

heterogeneous responses to one particular marker, thereby providing more accurate estimation of the force of infection. A recent follow-up study conducted on a cohort of children demonstrated that measuring Ab-responses to a selection of few Ags provide an accurate estimate of exposure for individuals and communities [32]. Therefore, further data analysis performed with multivariate analysis should provide added value. Moreover, presently it is not possible to exploit the full potential of the assay to take into account individual variation in Ab responses, as analysis of the SCR is still carried out Ag by Ag. Data analysis methods to combine Ab responses for estimation of SCRs exist [44] and are currently being extended in the context of this multiplex assay for detection of anti-*Plasmodium* Abs.

5 Conclusion

As many countries engage in malaria elimination it is crucial to improve the understanding of the effectiveness of additional malaria control tools and to be able to identify malaria hotspots for targeted and effective interventions in countries in an advanced elimination phase. With the current malariometric and entomological tools it is challenging to determine the malaria transmission rate in areas where prevalence of infection and incidence is decreasing. The multiplex assay measuring serological markers for malaria transmission was successfully implemented as the first in the Southeast Asian context. Moreover, it proved to be a reliable, fast and useful method for simultaneous detection of Abs against *Plasmodium* Ags of three different *Plasmodium* parasites, which has never been done before. The assay was used to screen almost 2,000 field blood spot samples in duplicate and proved to be very time efficient and informative. It was also possible to estimate differences in the force of malaria infection, by looking at the differences in SCR over time between the different Ags. For a better estimation of changes in the force of infection on long-term as well as short-term in areas with low malaria endemicity, more information on the longevity of the Ab responses is needed. The simultaneous detection of several Ags of different parasites offers an opportunity to look for a potential serological marker of very recent malaria transmission, which is the next step of this research. This will prove to be key knowledge for malaria elimination.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Sample collection of the survey was performed by the CNM team (National Centre for Parasitology, Entomology and Malaria Control), the ITM Antwerp team and the Institut Pasteur du Cambodge team in Ratanakiri Province in Cambodia. The study design and literature research has been performed at the ITM by KK, LD and MC. Protocols were written by KK, based on protocols from Institut Pasteur de Madagascar (IVW), verified by LC and validated by DM. Laboratorial work for the performance of the assay was performed by KK and LC, and KK executed the screening of the blood spot samples. Selection of the pool of control sera and the high positive control dilutions was done by KK, LD and LC. Data entry and analysis of the results were performed at the ITM by KK, and verified by LD, VS and MC. The first draft of the manuscript was written by KK. All authors read and approved the final manuscript.

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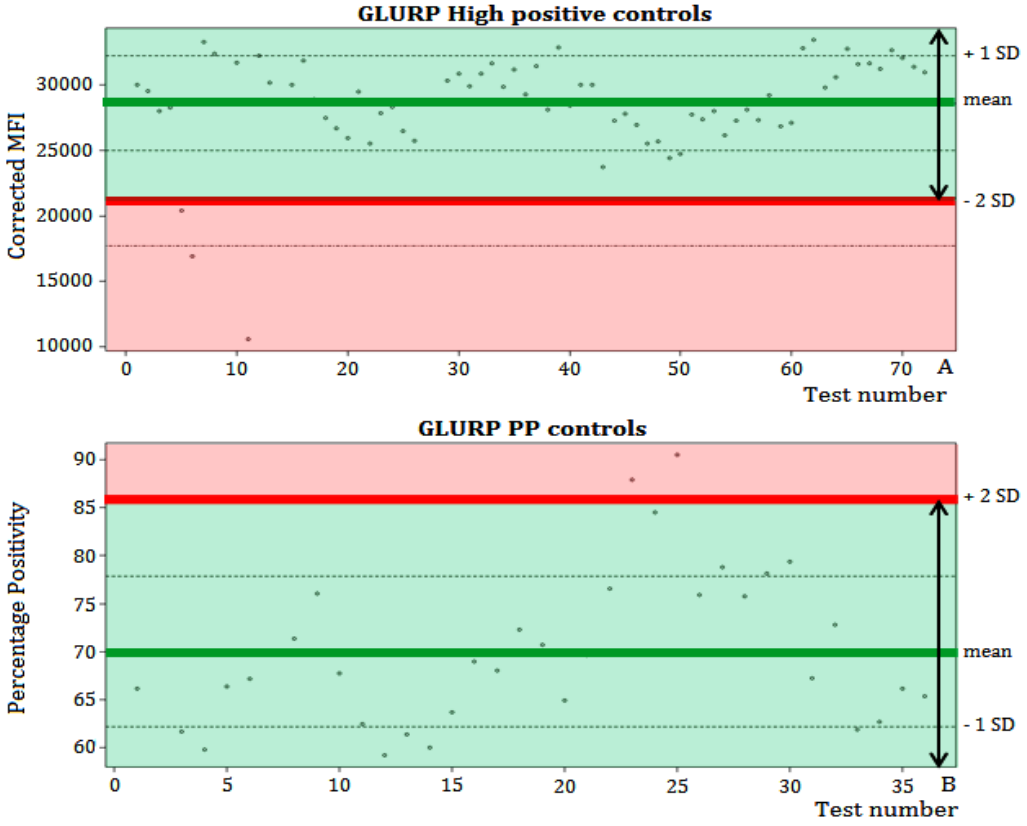
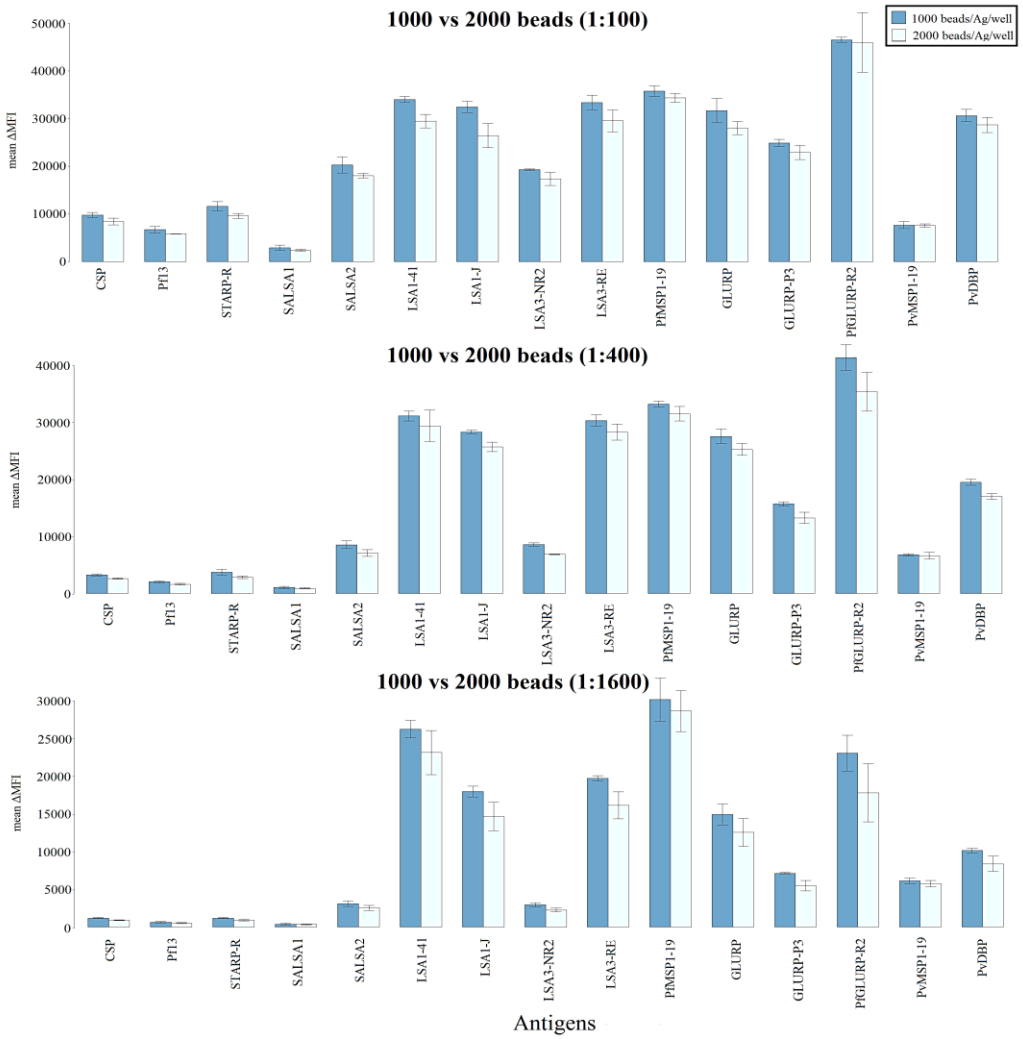
7 Additional files

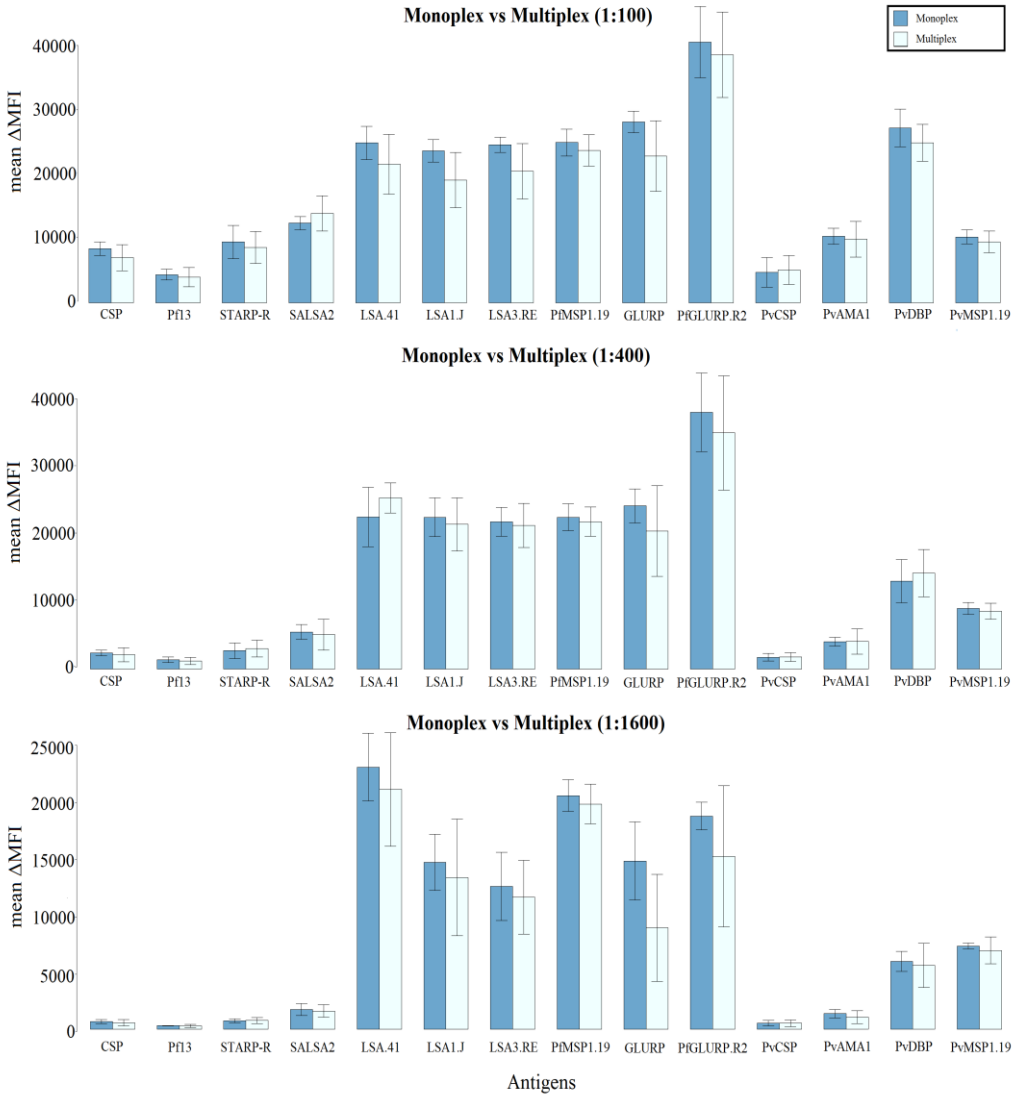
Additional file 1 Example of Levey Jenning Charts plotted for the quality control of the immunoassay used for screening the field bloodspot samples. Data analysis started with a quality control on the ΔMFI-values of the 100% positive control pool samples (A). The dots represent each positive control sera sample in duplicate per plate. If these dots fell out of the -2SD and +2SD (red area), these plates were rejected and re-analysed. The same quality control was also performed on the PP calculated from the 50% positive control pool samples per Ag (B). Based on the outcome of both graphs, plates were accepted or rejected and reanalysed.



Additional file 2 Comparison of the ΔMFI signals obtained from the immunoassay using 1,000 and 2,000 beads/Ag/well. The positive control pool was tested in the multiplex assay (dilutions 1:100, 1:400 and 1:1,600) for 1,000 beads/Ag/well (dark blue bars) and 2,000 beads/Ag/well (light blue bars). The error bars represent upper and lower limits of the 95% confidence intervals.



Additional file 3: Comparison of the ΔMFI signals obtained from the monoplex and multiplex immunoassay. Each Ag was tested in monoplex (each Ag separately) and in multiplex (all Ags pooled together) assay on the positive control pool (dilutions 1:100, 1:400 and 1:1600). The error bars represent upper and lower limits of the 95% confidence intervals.



Additional file 4: Results of the segmented regression analysis for assessing the stability of the antigen coupled beads per antigen. The breakpoints were estimated with the R-package 'segmented' and the ΔMFI-values obtained at Week (Wk) 4, Wk 8 and Wk16 were converted into percentages compared to the ΔMFI value obtained directly after coupling (Wk0).

Antigens	ΔMFI-range	dilutions	Breakpoint (weeks)	Percentage Wk4	Percentage Wk8	Percentage Wk16
CSP	539 - 8672.5	0.111111	3	103.3	91.4	75.7
		0.319444		107.7	90.4	81.3
		1.152778		114.3	83	85.3
Pf13	249 - 4880.5	0.111111	3	99	94.7	80.8
		0.319444		101	97.7	79.2
		1.152778		98.3	85.8	79.8
STARP-R	577 - 11355.5	0.111111	3	112.7	93.3	75.9
		0.319444		113.9	89.4	75.7
		1.152778		116.8	81.8	72.9
SALSA2	1488 - 17319	0.111111	3	104.1	99.8	87.9
		0.319444		110.9	100.2	93.5
		1.152778		119.2	96.9	105.2
LSA1-41	9657 - 27963.5	0.111111	7	94.5	100.6	62.8
		0.319444		108.4	102	77
		1.152778		104.8	106.8	84.7
LSA1-J	10206 - 25600.5	0.111111	6	93.5	103.7	65.6
		0.319444		118.1	107.8	84
		1.152778		116.5	103.7	88.4
LSA3-RE	9885 - 28685	0.111111	6	98.8	108.5	71.6
		0.319444		120.1	110.6	79
		1.152778		119.8	108.1	87.6
PfMSP1-19	18840 - 26888.5	0.111111	7	90.7	101.1	82.5
		0.319444		97.5	105.3	88.2
		1.152778		98.4	103.6	90.2
GLURP	6443 - 30340	0.111111	6	93.7	106.1	69
		0.319444		131.2	123.4	90.3
		1.152778		117.1	92.1	88.4
PfGLURP-R2	13995.5 - 41999	0.111111	7	94.6	99.3	59.8
		0.319444		115.7	106.2	69.9
		1.152778		115.2	93.7	75.1
PvCSP	452 - 6187.5	0.111111	3	76.1	74.4	56.8
		0.319444		76.5	60.7	55.5
		1.152778		75.4	61.9	60.3
PvAMA1	1353 - 13328.5	0.111111	3	112.1	105.7	89.9
		0.319444		111.4	102.8	89.3
		1.152778		122.5	101.1	103.9
PvDBP	4505 - 26142.5	0.111111	3	109.8	105.2	80.9
		0.319444		111.9	101.6	82.3
		1.152778		121.5	98.8	88.7
PvMSP1-19	4328 - 9115.5	0.111111	7	96.7	106.5	80.8
		0.319444		94.2	110.8	83
		1.152778		97.2	107.7	86.8

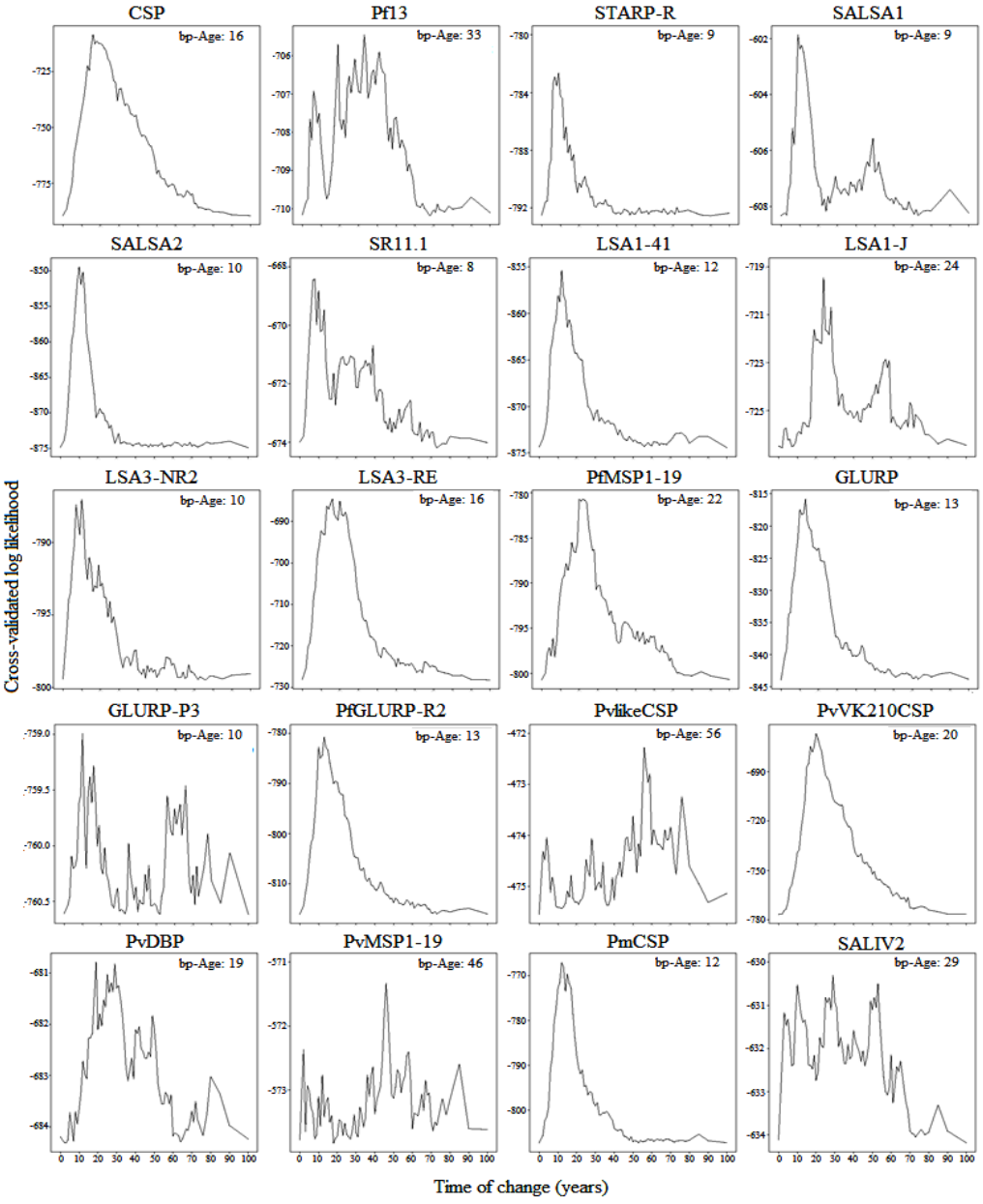
Additional file 5: Seroconversion (λ) and Seroreversion (ρ) rates estimated for each Ag by catalytic regression models allowing one (Model 1) and two (Model 2) SCRs.

Antigens	Model	Parameters	Estimate [95% CI]	Rho (ρ) [95% CI]	AIC[1]	LRT-test[2]
CSP	Model 1	λ	Not calculated	0.006 [0.003 - 0.01]	268	< 0.05
	Model 2	λ1 (≤ 16 years)	0.020 [0.012 - 0.029]		263	
		λ2 (> 16 years)	0.047 [0.034 - 0.059]			
Pf13	Model 1	λ	0.017 [0.008 - 0.026]	0.028 [-0.016 - 0.073]	227	> 0.05
	Model 2	λ1 (≤ 33 years)	0.018 [0.012 - 0.024]		228	
		λ2 (> 33 years)	0.014 [0.004 - 0.024]			
STARP-R	Model 1	λ	0.018 [0.010 - 0.025]	0.007 [-0.018 - 0.033]	247	> 0.05
	Model 2	λ1 (≤ 9 years)	0.009 [0.002 - 0.017]		245	
		λ2 (> 9 years)	0.020 [0.014 - 0.025]			
SALSA1	Model 1	λ	0.013 [0.007 - 0.020]	0.012 [-0.022 - 0.046]	212	> 0.05
	Model 2	λ1 (≤ 9 years)	0.007 [0.000 - 0.014]		212	
		λ2 (> 9 years)	0.015 [0.010 - 0.019]			
SALSA2	Model 1	λ	0.033 [0.023 - 0.043]	0.006 [-0.009 - 0.022]	294	< 0.05
	Model 2	λ1 (≤ 10 years)	0.016 [0.006 - 0.026]		288	
		λ2 (> 10 years)	0.039 [0.029 - 0.048]			
SR11.1	Model 1	λ	0.010 [0.005 - 0.016]	0.004 [-0.030 - 0.038]	198	> 0.05
	Model 2	λ1 (≤ 8 years)	0.007 [0.000 - 0.013]		199	
		λ2 (> 8 years)	0.011 [0.007 - 0.015]			
LSA1-41	Model 1	λ	0.049 [0.033 - 0.057]	0.006 [-0.007 - 0.020]	319	> 0.05
	Model 2	λ1 (≤ 12 years)	0.038 [0.026 - 0.050]		319	
		λ2 (> 12 years)	0.051 [0.038 - 0.065]			
LSA1-J	Model 1	λ	0.020 [0.008 - 0.033]	0.048 [-0.016 - 0.033]	240	> 0.05
	Model 2	λ1 (≤ 24 years)	0.021 [0.014 - 0.029]		243	
		λ2 (> 24 years)	0.018 [0.009 - 0.028]			
LSA3-NR2	Model 1	λ	0.040 [0.007 - 0.074]	0.128 [-0.031 - 0.286]	276	> 0.05
	Model 2	λ1 (≤ 10 years)	0.042 [0.022 - 0.062]		277	
		λ2 (> 10 years)	0.039 [0.025 - 0.054]			
LSA3-RE	Model 1	λ	0.070 [0.056 - 0.084]	0.002 [-0.003 - 0.006]	296	< 0.05
	Model 2	λ1 (≤ 16 years)	0.058 [0.042 - 0.074]		294	
		λ2 (> 16 years)	0.085 [0.063 - 0.107]			
PfMSP1-1	Model 1	λ	0.072 [0.055 - 0.088]	0.006 [-0.005 - 0.017]	327	> 0.05
	Model 2	λ1 (≤ 22 years)	0.070 [0.056 - 0.085]		329	
		λ2 (> 22 years)	0.076 [0.047 - 0.104]			
GLURP	Model 1	λ	0.043 [0.032 - 0.054]	0.003 [-0.007 - 0.013]	303	> 0.05
	Model 2	λ1 (≤ 13 years)	0.035 [0.023 - 0.046]		302	
		λ2 (> 13 years)	0.051 [0.037 - 0.064]			
GLURP-P3	Model 1	λ	0.016 [0.008 - 0.024]	0.016 [-0.020 - 0.053]	237	> 0.05
	Model 2	λ1 (≤ 10 years)	0.014 [0.004 - 0.023]		239	
		λ2 (> 10 years)	0.017 [0.011 - 0.022]			
PfGLURP-I	Model 1	λ	0.049 [0.038 - 0.061]	0.002 [-0.006 - 0.011]	306	< 0.05
	Model 2	λ1 (≤ 13 years)	0.039 [0.027 - 0.051]		304	
		λ2 (> 13 years)	0.059 [0.044 - 0.074]			
PvlikeCSP	Model 1	λ	0.008 [0.001 - 0.015]	0.040 [-0.046 - 0.126]	149	> 0.05
	Model 2	λ1 (≤ 56 years)	0.008 [0.005 - 0.012]		151	
		λ2 (> 56 years)	0.008 [-0.005 - 0.022]			
PvVK210C	Model 1	λ	Not calculated	0.005 [-0.005 - 0.015]	218	< 0.05
	Model 2	λ1 (≤ 20 years)	0.009 [0.005 - 0.014]		209	
		λ2 (> 20 years)	0.033 [0.021 - 0.044]			
PvDBP	Model 1	λ	0.017 [0.006 - 0.028]	0.047 [-0.021 - 0.115]	222	> 0.05
	Model 2	λ1 (≤ 19 years)	0.018 [0.108 - 0.025]		224	
		λ2 (> 19 years)	0.016 [0.007 - 0.025]			
PvMSP1-1	Model 1	λ	0.011 [0.002 - 0.019]	0.039 [-0.044 - 0.121]	179	> 0.05
	Model 2	λ1 (≤ 46 years)	0.011 [0.006 - 0.015]		180	
		λ2 (> 46 years)	0.015 [-0.006 - 0.035]			
PmCSP	Model 1	λ	0.017 [0.011 - 0.023]	0.005 [-0.011 - 0.020]	241	< 0.05
	Model 2	λ1 (≤ 12 years)	0.010 [0.004 - 0.016]		235	
		λ2 (> 12 years)	0.025 [0.018 - 0.032]			
SALIV2	Model 1	λ	0.019 [0.003 - 0.035]	0.083 [-0.042 - 0.208]	210	> 0.05
	Model 2	λ1 (≤ 29 years)	0.019 [0.012 - 0.026]		212	
		λ2 (> 29 years)	0.019 [0.002 - 0.037]			

[1]AIC: Akaike?s Information Criterion

[2]p-value from likelihood ratio test

Additional file 6: V-fold cross-validated plots with age-varying seroconversion rates.
These plots were used to estimate the optimal breakpoint in age per antigen. The VFCV randomly partionate the data into a validation set and a training set. Then the cross-validation is repeated V-times on the training set and a least once on the validation set. All V-results are avaraged and gave a single estimation.



A MULTIPLEX IMMUNOASSAY TO IMPROVE THE UNDERSTANDING OF THE EFFECTIVENESS OF THE ADDITIONAL USE OF TOPICAL REPELLENT ON MALARIA INCIDENCE IN A COMMUNITY BASED TRIAL

THIS STUDY WAS PUBLISHED AS:

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Author's contributions:

LD, VS, DM and MC designed the trial. VS, SH, LD, SK, KVR, CG, SM, SU, SS, ST, DM, MC organized the field work. SH, SK, VS, LD, CG and KVR collected the data. LD, LC, SK, NK, KK, DM organized and performed the lab work. VS performed the statistical analysis. VS, LD, CG, SH, KPG, DM, MC wrote the paper. MC is guarantor.

This chapter is built upon the paper written by Vincent Sluydts, because this PhD is based on the serological data collected during the MalaRest project. KK (the PhD thesis author) was responsible for executing serology as the secondary outcome of the MalaResT project. Therefore, in the following chapter text written in *italic* is cited from the original paper, while all other text is own additional value.

1. Background

During the past decade, the scaling-up of long-lasting insecticidal nets (LLIN) and, to a lesser extent indoor residual spraying (IRS) has contributed to a worldwide decline in the malaria burden. These highly effective vector control tools target mosquitoes resting or feeding indoors at night. Impregnated bednets inhibit blood feeding and induce a mass killing effect on vector populations assuring the required community protection against malaria. Nonetheless, some malaria vectors show early and outdoor biting behavior, causing community protection to remain incomplete as a proportion of the vector population is not exposed to the insecticides. To tackle this remaining or residual transmission, additional vector control tools are required, particularly in settings where malaria control programmes move towards elimination (paragraph cited from Sluydts et al., [1]).

Topical repellents possess the potential to target residual transmission. Entomological evidence has shown that repellents provide personal protection against malaria. However, the efficacy of large-scale coverage of repellents on community protection against malaria remains unverified. Previous studies have assessed the efficacy of topical repellents against malaria infection using individual randomised designs, household randomised trials, and several community based studies. A meta-analysis concluded that topical repellents are ineffective in preventing malaria morbidity, with substantial differences in study design among the studies included in the final analysis (nine for Plasmodium falciparum and seven for Plasmodium vivax). The main limitation of these studies lies in the diversion of vectors from individual or household repellent-users to non-users, spill-over effects because of geographical contiguous communities, or follow-up periods immediately after the onset of the intervention. A large-scale, cluster-randomized trial attempting complete and continuous coverage of the vector control tool in the intervention group over two transmission seasons was expected to have a more uniform effect on malaria transmission. Moreover, wide-scale use of repellents in the community (cluster) can induce a reduction of the malaria burden by reducing human-vector contact, the so-called, mass-effect (paragraph cited from Sluydts et al., [1]).

In the Greater Mekong subregion, a diverse community of malaria vectors is present, several of which are known to feed outdoors and before nightfall, compromising the effectiveness of domicile vector control tools such as of long-lasting insecticidal nets and indoor residual spraying. We hypothesized that the synergy between indoor nighttime protection of a long-lasting insecticidal net and outdoor evening protection

of a topical repellent would further reduce the malaria transmission intensity, by affecting the vector population remaining outdoors. This study in which this PhD is embedded aimed to evaluate the epidemiological efficacy of a highly effective topical repellent in addition to long-lasting insecticidal nets in the province of Ratanakiri, Cambodia, a region where vectors are known to show outdoor and early evening biting behaviour (paragraph cited from Sluydts et al., [1]).

The principal indicator of this study was the prevalence of parasite carriers measured by PCR techniques in which the difference in prevalence between the two arms was attributed to the residual transmission avoided by the use of repellents. Next, the incidence of symptomatic malaria as reported by Village Malaria Workers was considered. Changes in sero-prevalence shown in this chapter were estimated for malaria incidence, and used as secondary indicator for comparing the force of infection between both arms. Data on malaria parasite incidence by using serological markers offers the advantage over parasite prevalence in that anti-*Plasmodium* antibodies can persist for months after infection and have been suggested as good indicators of malaria transmission dynamics [2]. Therefore an assay based on the use of multiple serological markers that reflects the different stages within the life-cycle [3, 4] to estimate the exposure to different *Plasmodium* species was implemented and applied on samples from Ratanakiri, Cambodia. Antigens with a relative short half-life are privileged.

2. Material and Methods

2.1 Study design

This study is a cluster randomized controlled trial (the MalaResT project, NCT01663831) conducted in the most endemic (117 out of 240) villages in Ratanakiri province located in the north-east of Cambodia. Up to now, malaria is still a major cause of mortality and morbidity in Cambodia, of which Ratanakiri is a province with highest diagnostic-based malaria incidence rates (22,2 per 1,000 individuals in 2007) [5, 6] with a peak from June till October (rainy season). Ethnic minorities, temporary migrants, settlers in forested areas and plantation workers are among the population most at risk to *Plasmodium* infections in the Western Pacific region [6].

From the 117 villages four villages were omitted because they cannot be reached during the rainy season. Therefore, 112 villages with highest incidence distributed in 98 clusters (approximately 48,000 inhabitants) are included in this study. These 98 clusters were randomly appointed to a control arm (high coverage with LLIN), and to

an intervention arm (high LLIN coverage + daily use of topical repellents) (Figure 1). Both arms consisted of 49 clusters. For the intervention arm the topical repellents where distributed every two weeks between March and December and consisted of 10% Picardin lotion and 20% spray. Five cross-sectional surveys were organized during two years (2012 – 2013) [1, 5, 7]. The first survey was the pre-trial survey, conducted in February 2012 and used to obtain baseline information on malaria prevalence at cluster level [1]. The four additional surveys were performed at the beginning and the end of the rainy season (Survey 1 in April 2012, Survey 2 in October 2012, Survey 3 in March 2013, and Survey 4 in October 2013 (Figure 1)). For each survey a random selection of 65 participants per cluster was made (between 2 - 99 years old) for a two-day sample collection. Blood samples were collected by finger prick on a filter paper [1, 5, 7]. After collecting the blood samples on a filter paper these were immediately analyzed by real-time PCR to check the parasite-prevalence [1, 5, 7]. At the end of this trial there were three different outcomes.

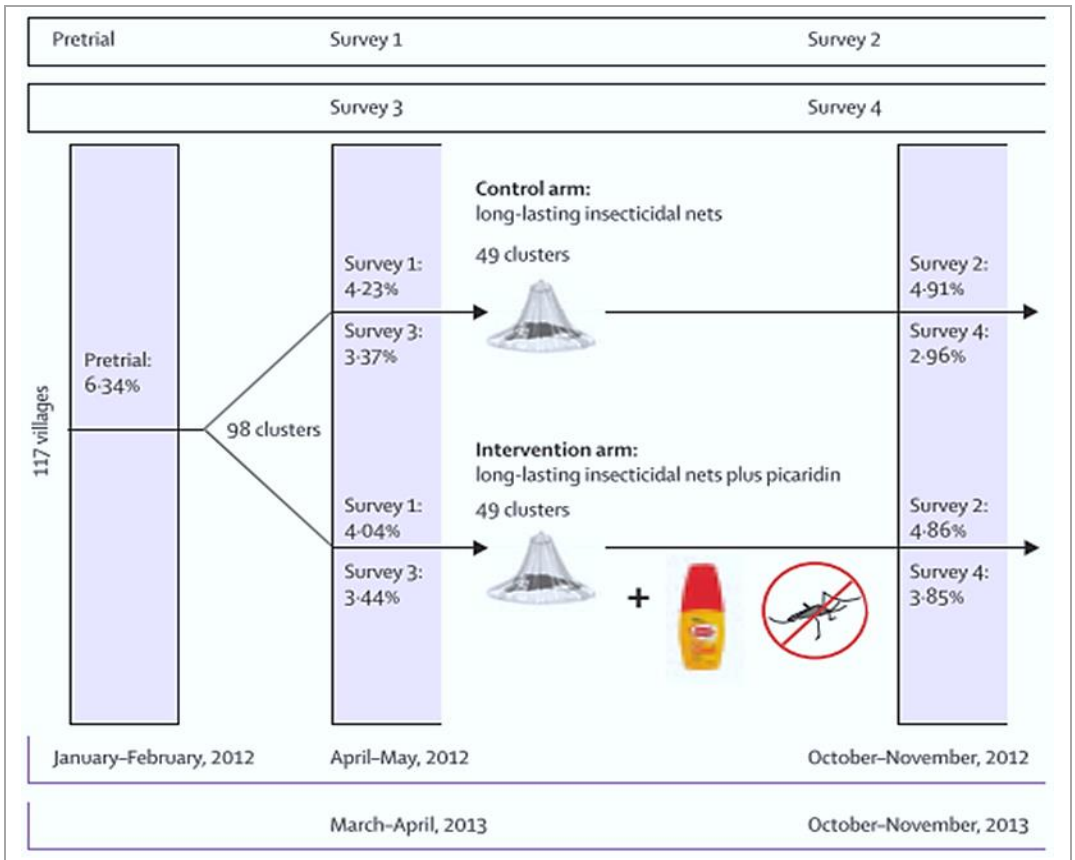

Figure 1: This figure is derived from the paper published by Sluydts et al. (2016) [1]. Design of the cluster randomised trial with two study groups. Grey boxes indicate the timing of the surveys during which the primary endpoint (PCR prevalence) was obtained.

2.1.1 Real-time PCR

The primary outcome is the malaria prevalence based on the detection of the *Plasmodium cytochrome b gene* by using a real-time PCR assay. The first step was to screen the blood samples for malaria parasite using genus-specific primers. Within this step samples were screened on being malaria positive or negative, which allows for treatment of the positive cases within 24-48 hours after sampling. In the next step a nested real-time PCR assay was used to identify the different malaria species *(P. falciparum, P. vivax, P. malariae and P. ovale)* on the positive samples from the first step. These tests have been performed in an "in-house" designed mobile laboratory in the field [8]. For the primary outcome no significant difference was seen in PCR *Plasmodium* prevalence between control and intervention in 2012 and 2013 (Figure 1).

2.1.2 Passive case Detection

The secondary outcome of the MalaResT project is based on malaria incidence by Passive Case Detection. Passive Case Detection is based on passive diagnosis carried out by village malaria workers (VMW), and malaria diagnosis by microscopy and RDTs in public health facilities (PHF). The data are subtracted from the malaria information system (MIS). MIS does not report the data from private sectors and asymptomatic cases [9]. The annual parasite incidence (API) has been estimated according to the following formula: $API = \frac{no.Parasitologically\ confirmed\ malaria\ cases/year}{Population\ at\ risk} * 1000$ [1, 7]. APIs were estimated for *Plasmodium* species *P. falciparum* and *P. vivax*. The results did not show any significant differences ($p > 0.05$) between the control and intervention arm among the entire study population.

2.1.3 Serology

For the serology data on malaria parasite incidence was used. These samples were both tested on the PCR as well as for serological markers for malaria. This offers the advantage over parasite prevalence in that anti-*Plasmodium* Abs can persist for months after infection and have been suggested as indicators of malaria transmission dynamics. Changes in Ab-titers can be an alternative way to estimate the malaria incidence by comparing the optical density value of two successive tests on the same individual. A multiplex immunoassay for the simultaneous detection of Abs against different malaria Ags was used.

2.2 Serological markers used in the multiplex immunoassay

The multiplex assay uses paramagnetic beads in order to detect the antibody response to multiple antigens simultaneously [10]. The principle of this technique is based on an

ELISA, with the difference that antigens are coated on magnetic beads instead of on a well. In this study the following peptides and recombinant proteins are used: *P. falciparum* specific antigens (CSP, Pf13, STARP-R, SALSA1, SALSA2, SR11.1, LSA1-41, LSA1-J, LSA3-NR2, LSA3-RE, PfMSP1-19, GLURP, GLURP-P3, PfGLURP-R2), *P. vivax* specific antigens (Pvlike-CSP, PvVK210CSP, PvVK247CSP, PvCSP (chimera), PvAMA1, PvEBP, PvDBP, PvMSP1-19), *P. malariae* specific peptide (PmCSP) and two peptides specific for the *An. gambiae* saliva protein (SALIV1, SALIV2). These antigens were coupled to the beads as described by Kerkhof et al., [4]. In brief, in the first step of the coupling process the carboxyl groups on the beads-surface are activated to form an unstable reactive group [11], which binds to the primary amines on the antigens. After coupling the different coloured beads to their antigens, the coupling process is confirmed by testing the coupled beads on well characterized control sera. Hereafter, the multiplex immunoassay is carried out as described by Kerkhof et al., [4]. In every test, negative and positive controls are included. Eluted bloodspots (see further) are incubated with the mixture of coated beads, to capture the specific anti-*Plasmodium* antibodies, which are then incubated with a phycoerythrin-labeled IgG specific secondary antibody. In order to obtain optimal results, the immunoassay is performed in duplicate on all samples. In a final step, the plates are read with the MAGPIX® instrument (Luminex Corporation). The MAGPIX® will read a minimum amount of 400 beads per spectral address and yields two data-outputs that represents the median fluorescence intensity (MFI) value and the bead count [3, 4, 12, 13]. A quality control was performed on the positive controls, to ensure that all tests were performed in equal optimal conditions. Results of the individual samples are expressed as percentage positivity ($\frac{\Delta MFI\ samples\ (Ag1)}{\Delta MFI\ High\ positive\ control\ (Ag1)}$**100%*) calculated from the low positive control pool samples [4].

The multiplex assay was applied on a random selection of 6,991 samples from survey 2 (3,498 samples) and survey 4 (3,493 samples). Analysis of these samples focus on the comparison between the control and intervention arm within the MalaResT project.

2.3 Data analysis

Generalized linear mixed models (geeglm) was carried out to compare the control and intervention arm [14]. This analysis was based on randomly chosen samples from survey 2 (2012) and survey 4 (2013). The GEE approach is commonly used to estimate effects on population level [14]. The GEE-model was generated using a gaussian error distribution on the transformed MFI data using the natural logarithm. A non-adjusted GEE-model and an adjusted GEE-model (Age (continuous) and Gender (categorical)) were carried out. In both analysis within cluster correlations were taken into account

[15]. This model estimates the odd's ratio (OR) as well as the p-values per Ag per survey. To account for multiple comparisons (42 different measures) a Bonferroni correction was applied ($adjusted\ p-value$: $\frac{0.05\ (p-value)}{42\ tests} = 0.011$) with p-values below 0.011 considered statistically significant [16, 17]. All analysis were programmed and analysed with R software package version 3.1.0. [18].

2.4 Ethical clearance

The study protocol was reviewed and approved by the Cambodian National Ethics Committee on Health Research (Approval 265 NECHR), the Institutional Review Board of the Institute of Tropical Medicine Antwerp (Approval IRB/AB/ac/154) and the Ethics Committee of the University of Antwerp (Approval B300201112714). Gatekeepers provided informed written consent for the participation of their village. The survey participant or his/her parents or guardian provided informed written consent for individual participation.

3. Results

3.1 Serological outcome of comparison between control and intervention arm

No significant difference between the two study arms (p>0.05) is observed for all twenty-one Ags (Table 1). As this is a comparative analysis (GEE model), it should be adjusted for confounding in this case for Age (continuous) and Gender (categorical). After adjusting for age and gender, still no significant difference (p>0.05) is seen between the two arms.

Non-adjusted and adjusted (Table 1) OR with confidence interval (CI) for the comparison of the control and intervention group were estimated. The outcome of the OR for both analyses is for all Ags not significantly different from 1. An OR of 1 implies there is no difference between the two arms of the study.

Table 1: P-values indicate differences in seroprevalence between control and intervention arm. It represents both non-adjusted and age and gender adjusted analysis of log transformed Median Fluorescence Intensity data for all *Plasmodium* species. Bonferroni corrected p-values are estimated with GEE-model as well as the incidence rate ratios' and confidence intervals taking into account within cluster correlation. No significant difference between the control and intervention arm.

			Mean log MFI		Non-adjusted analysis		Adjusted analysis	
	Antigen	Species	Control	Intervention	IRR (95% CI)	p-value	IRR (95% CI)	p-value
Survey 2 (2012)	CSP	Pf	7.81	7.86	1.10 (0.85 - 1.43)	0.45	1.04 (0.90 - 1.21)	0.59
	Pf13	Pf	8.23	8.20	1.04 (0.84 - 1.29)	0.71	1.01 (0.86 - 1.17)	0.89
	STARP.R	Pf	7.41	7.40	1.06 (0.82 - 1.38)	0.65	1.03 (0.87 - 1.22)	0.75
	SALSA2	Pf	8.08	8.15	1.17 (0.86 - 1.58)	0.32	1.09 (0.89 - 1.33)	0.39
	SR11.1	Pf	8.79	8.75	1.01 (0.77 - 1.33)	0.94	1.00 (0.83 - 1.20)	0.99
	LSA1.41	Pf	8.78	8.75	1.00 (0.74 - 1.36)	0.99	0.99 (0.81 - 1.22)	0.94
	LSA1.J	Pf	7.13	7.09	1.01 (0.81 - 1.26)	0.93	1.00 (0.86 - 1.17)	1.00
	LSA3.RE	Pf	9.56	9.67	1.17 (0.78 - 1.75)	0.46	1.09 (0.84 - 1.41)	0.51
	PfMSP1.19	Pf	7.66	7.61	0.99 (0.78 - 1.25)	0.90	0.98 (0.84 - 1.16)	0.84
	GLURP	Pf	8.95	9.02	1.11 (0.80 - 1.56)	0.53	1.04 (0.84 - 1.30)	0.71
	PfGLURP.R2	Pf	9.67	9.70	1.06 (0.74 - 1.52)	0.76	1.01 (0.80 - 1.28)	0.93
	PvVK210CSP	Pv	7.21	7.25	1.08 (0.87 - 1.35)	0.49	1.02 (0.91 - 1.16)	0.71
	PvVK247CSP	Pv	6.99	7.00	1.04 (0.87 - 1.24)	0.68	1.00 (0.90 - 1.11)	0.99
	PvCSP	Pv	8.47	8.46	1.02 (0.86 - 1.21)	0.87	1.01 (0.89 - 1.13)	0.94
	PvAMA1	Pv	8.97	9.02	1.08 (0.86 - 1.36)	0.52	1.04 (0.89 - 1.22)	0.63
	PvEBP	Pv	10.08	10.13	1.07 (0.74 - 1.54)	0.72	1.02 (0.80 - 1.31)	0.87
	PvDBP	Pv	8.02	8.08	1.10 (0.90 - 1.34)	0.34	1.06 (0.93 - 1.21)	0.40
	PvMSP1.19	Pv	7.68	7.67	1.05 (0.83 - 1.33)	0.67	1.03 (0.88 - 1.21)	0.74
	PmCSP	Pm	8.18	8.17	1.05 (0.83 - 1.33)	0.71	1.02 (0.87 - 1.19)	0.82
	SALIV1	Anopheles	7.82	7.69	0.88 (0.67 - 1.15)	0.35	0.91 (0.76 - 1.09)	0.31
	SALIV2	Anopheles	7.74	7.65	0.90 (0.68 - 1.20)	0.49	0.89 (0.67 - 1.18)	0.42
Survey 4 (2013)	CSP	Pf	7.83	7.91	1.16 (0.89 - 1.50)	0.28	1.08 (0.92 - 1.26)	0.34
	Pf13	Pf	8.36	8.32	1.03 (0.83 - 1.29)	0.78	1.02 (0.98 - 1.19)	0.80
	STARP.R	Pf	7.45	7.36	1.01 (0.78 - 1.29)	0.96	1.00 (0.85 - 1.18)	1.00
	SALSA2	Pf	8.15	8.08	1.06 (0.80 - 1.40)	0.70	1.04 (0.86 - 1.25)	0.72
	SR11.1	Pf	8.95	8.84	1.00 (0.76 - 1.31)	0.99	0.99 (0.82 - 1.20)	0.94
	LSA1.41	Pf	8.57	8.74	1.25 (0.94 - 1.65)	0.12	1.17 (0.96 - 1.41)	0.11
	LSA1.J	Pf	7.25	7.15	0.97 (0.80 - 1.17)	0.72	0.98 (0.86 - 1.12)	0.72
	LSA3.RE	Pf	9.33	9.57	1.31 (0.89 - 1.94)	0.18	1.18 (0.90 - 1.54)	0.24
	PfMSP1.19	Pf	7.87	7.66	0.84 (0.66 - 1.07)	0.15	0.88 (0.74 - 1.05)	0.15
	GLURP	Pf	8.81	9.00	1.23 (0.89 - 1.70)	0.20	1.16 (0.93 - 1.45)	0.20
	PfGLURP.R2	Pf	9.42	9.59	1.23 (0.87 - 1.73)	0.24	1.15 (0.90 - 1.45)	0.27
	PvVK210CSP	Pv	7.34	7.41	1.12 (0.91 - 1.38)	0.28	1.06 (0.94 - 1.21)	0.34
	PvVK247CSP	Pv	7.10	7.15	1.10 (0.93 - 1.30)	0.24	1.06 (0.95 - 1.18)	0.32
	PvCSP	Pv	8.61	8.63	1.04 (0.87 - 1.23)	0.68	1.02 (0.91 - 1.15)	0.69
	PvAMA1	Pv	9.08	9.10	1.09 (0.89 - 1.33)	0.42	1.06 (0.92 - 1.22)	0.46
	PvEBP	Pv	9.88	10.15	1.38 (0.97 - 1.97)	0.07	1.26 (0.99 - 1.59)	0.06
	PvDBP	Pv	8.14	8.20	1.17 (0.94 - 1.45)	0.16	1.12 (0.96 - 1.30)	0.14
	PvMSP1.19	Pv	7.86	7.76	1.00 (0.79 - 1.27)	0.97	1.00 (0.85 - 1.18)	0.99
	PmCSP	Pm	8.35	8.32	1.07 (0.85 - 1.36)	0.57	1.05 (0.89 - 1.23)	0.57
	SALIV1	Anopheles	8.09	7.88	0.84 (0.63 - 1.13)	0.25	0.89 (0.73 - 1.09)	0.26
	SALIV2	Anopheles	8.01	7.81	0.83 (0.62 - 1.12)	0.23	0.84 (0.62 - 1.13)	0.25

4. Discussion

This cluster randomized trial, designed to detect population-level rather than individual effects, did not show a reduction in malaria infection and disease in the intervention group. When compared with earlier cluster-based intervention studies of topical repellents this trial was scaled up by a factor of six, incorporating 49 clusters (communities) in each treatment group. All existing homesteads had continuous access to repellents and received long-lasting insecticidal nets. Given the large number of randomized clusters, any confounder that might affect the measured outcomes was likely to be balanced between the two study groups, including the proportion of P. vivax relapses, which are not affected by vector control tools (paragraph cited from Sluydts et al., [1]).

The study, which was done in a multidisciplinary setting, aimed to cover the epidemiological, entomological, and anthropological aspects of the trial, obtained three epidemiological endpoints: PCR prevalence, PCR incidence, and PCR malaria exposure measured by serological markers. Similar trends were observed in these three outcomes and no indication for a supplementary protective effect provided by mass-use of topical repellents in addition to long-lasting insecticidal nets on malaria prevalence was found. These results are in line with a recent meta-analysis of household and small community randomized trials in which repellents were found unlikely to provide effective protection against malaria. (paragraph cited from Sluydts et al., [1]).

This cluster randomized trial, designed to assess the epidemiological efficacy of community-wide use of topical repellents in addition to long lasting insecticidal nets, did not show a reduction in malaria infection and disease in the intervention group. Although individual protective efficacy of repellents against malaria vectors remains guaranteed, the results of this cluster-based trial support the conclusion that community-wide distribution of highly effective topical repellents might not contribute to a further decrease of malaria endemicity in a pre-elimination setting. Daily compliance and appropriate use of the repellents, achieved under optimum trial conditions with sufficient resources to promote and distribute the repellent product, remain the main obstacles. We conclude that mass distribution of topical repellents in addition to long-lasting insecticidal nets has no added public health value in preventing malaria in low endemic countries from the greater Mekong subregion (paragraph cited from Sluydts et al., [1]).

The serological outcomes of both study arms were estimated and compared to each other. Within this analysis no significant difference was observed, meaning that there is no proof that adding repellent on top of the LLINs has an effect on reducing malaria prevalence. These findings are in line with the preliminary results from the PCR prevalence and Passive Case Detection that also showed no significant difference between control and intervention arm [1]. As such serology provides no additional information, however, it does provide very strong evidence of no effect of the intervention that one might expect.

A possible explanation is that people might not use the vector control tools properly and consistent. This can affect the effectiveness of nets and repellents. Therefore, it would be useful to collect more data on human behavior and use a statistical model that can predict this data [19].

5. Conclusion

As Cambodia engages in malaria elimination by 2025, it is crucial to understand the effectiveness of the used malaria (vector) control tools. As previous used methods (real-time PCR and Passive Case detection) did not detect a difference between the control and intervention arm in the Ratanakiri province, it was useful to see if serology could make a difference. The findings of this evaluation are in line with the previous evaluations, that intervention has no effect on malaria prevalence, as no difference is seen between both arms.

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SEROLOGICAL MARKERS TO MEASURE RECENT CHANGES IN MALARIA AT POPULATION LEVEL IN CAMBODIA

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Abstract

Background: Serological markers for exposure to different *Plasmodium* species have recently been used in multiplex immunoassays based on the Luminex technology. However, interpretation of the assay results requires consideration of the half-life of specific antibodies against these markers. Therefore, the aim of the present study was to document the half-life of malaria specific serological makers, as well as assessing the sensitivity of these markers to detect recent changes in malaria exposure.

Methods: A recently developed multiplex immunoassay was used to measure the intensity of Ab-responses against 19 different *Plasmodium* specific antigens, covering different human malaria parasites and two vector saliva antigens. Therefore, 8,439 blood samples from five cross-sectional surveys in Ratanakiri, Cambodia, were analysed. These involve a random selection from two selected surveys, and an additional set of blood samples of individuals that were randomly re-sampled three, four or five times. A generalized estimating equation model and linear regression models were fitted on log transformed antibody intensity data.

Results: Results showed that most (17/21) Ab-responses are higher in PCR positive than PCR negative individuals. Furthermore, these antibody-responses follow the same upward trend within each age group. Estimation of the half-lives showed differences between serological markers that reflect short- (seasonal) and long-term (year round) transmission trends. Ab levels declined significantly together with a decrease of PCR prevalence in a group of malaria endemic villages.

Conclusion: For *Plasmodium falciparum,* antibodies against LSA3.RE, GLURP and Pf.GLURP.R2 are most likely to be a reflexion of recent (range from six to eight months) exposure in the Mekong Subregion. PvEBP is the only *Plasmodium vivax* Ag responding reasonably well, in spite of an estimated Ab half-life of more than 1 year. The use of Ab intensity data rather dichotomizing the continuous Ab-titre data (positive *versus* negative) will lead to an improved approach for serological surveillance.

Keywords: Malaria, serological markers, malaria transmission, half life

1. Background

Despite a considerable decline, the malaria burden in the Greater Mekong Subregion is still high and has a major impact on public health, especially in some specific regions within countries [1]. With 68,000 malaria cases in 2013 and an annual parasite incidence rate of 4.6 per 1,000 persons, malaria is the main single-cause infectious disease in Cambodia [2]. From 2000-2015, Cambodia has achieved a reduction of >75% in malaria case incidence [3], resulting in very low and heterogeneous malaria transmission clustered in hotspots and hotpops [2, 4, 5]. When aiming for malaria elimination, these focused areas of low malaria transmission pose considerable challenges for epidemiological surveillance and evaluation of control and elimination measures [6].

In malaria elimination areas, detection and surveillance of persisting malaria transmission is key for focussing interventions [7]. Traditional techniques for determining malaria transmission intensity include entomological inoculation rates (EIR) and parasite prevalence (PP) estimates [8]. However, in low transmission areas, the EIR and PP lack sensitivity, due to very low numbers of parasite-positive samples, both in mosquitoes and humans [9, 10].

Alternatively, serology is believed to be more informative in obtaining epidemiological information in malaria control programmes. In particular, serological markers can be used as a proxy for *Plasmodium* transmission intensity in low endemic areas [11] where parasite carriage is reduced and vector populations persevere [12]. More specifically this corresponds to the measurement of the force of infection (FOI), reflecting a rate at which susceptible individuals acquire an infection per year in a given area [9]. Where the EIR and PP mainly focus on the presence or absence of the infection [11] serology focuses on antibodies (Abs) that remain in the blood longer than the parasite. This is profitable in measuring host-parasite contact, and may provide information on recent or past malaria exposure, but only in case the half-lives of the Abs are known [11, 13, 14].

From an immunological point of view, partial protective immunity to malaria is built up after recurrent infections, typically over a period of several years of exposure. Hence, different types of memory are expected based on the frequency of short- (several months) and long-lived plasma cells (years) [15]. In areas of seasonal malaria transmission, it is assumed that short-lived plasma cells appear early in life and long-lived plasma cells later on [16], which can be used to understand the measurements of Ab half-lives [16]. It is important to take into account the different parasite life stages

(skin, liver and early blood stages) as well [17]. Producing Abs against sporozoites or merozoites is challenging, due to the short time that sporozoites need to reach and penetrate liver cells, and for merozoites to reinvade erythrocytes. Strong Ab-responses are expressed against early blood stage Ags, which can be more useful in estimating cumulative exposure [12, 17]. Even though it is difficult to determine the half-life of Abs, some studies have already studied the Ab persistence [18–20].

The aim of the current study is to document the half-life of IgG-Abs against 21 Ags from specific malaria parasites (*Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae*) and saliva of vectors as well as assessing the sensitivity of the markers to detect recent changes in malaria exposure. Methods to estimate the half-life of serological markers are still in its infancy, therefore, to reach this goal, three different approaches [13, 21] were applied directly on continuous Ab intensity values (Figure 1) instead of using (arbitrary) thresholds to distinguish positives from negatives [22]. First, a comparison of the Ab-intensity between PCR-positive and PCR-negative samples was performed. Secondly, the half-life of the serological markers was documented by analysing sequential samples. Thirdly, the Ab-responses were examined in relation to the PCR endemicity. As there is no golden standard, the use of multiple approaches will improve the robustness of the results. As such, serological markers that perform best for all three approaches are considered to be most promising in reflecting recent exposure.

2. Methods

2.1 Malaria transmission patterns in the study area

Ratanakiri is the highest malaria endemic province of Cambodia. Malaria transmission occurs during the rainy season, between April/May and October/November. Over the years a major decline in malaria transmission had been observed in this province: In 2000, the recorded annual parasite incidence in Ratanakiri was 11/1,000 inhabitants, whereas [23, 24] in 2012 an average incidence of 3.1/1,000 inhabitants were reported [4]. The observed decline is attributed to the performance of the National Malaria Control Programme (high long-lasting insecticidal net coverage and improved case management) but also to environmental changes (deforestation) impacting the main vector *(Anopheles dirus)* [2].

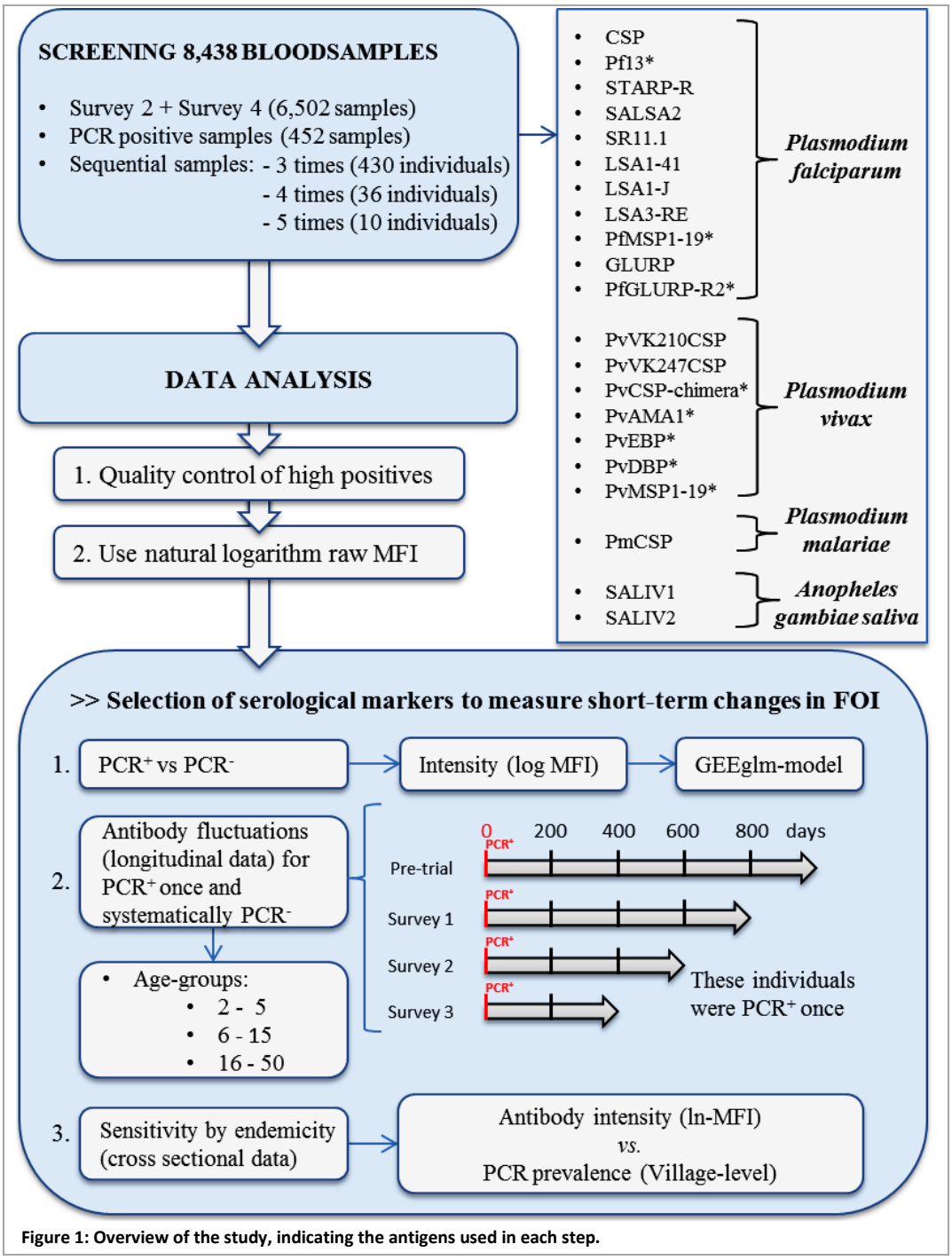

Figure 1: Overview of the study, indicating the antigens used in each step.

2.2 Samples

Serum samples used for this study were collected during the cluster randomized trial organized within the MalaResT project, NCT01663831, that aimed to evaluate the use of topical repellents as added control measure to long-lasting insecticidal nets for prevention of malaria [4, 5] in Ratanakiri province, Cambodia. The project took place in the 98 most endemic clusters in Ratanakiri (88 single villages and 25 neighbouring villages grouped into 10 clusters) [25]. These 98 clusters were split into two arms, a control (long-lasting insecticidal nets) and an intervention arm (long-lasting insecticidal nets and the topical repellent picaridin). During two consecutive years (2012/2013) individuals were sampled by collecting two drops of blood on filter paper through a finger prick. To obtain baseline information on malaria prevalence at cluster level, the first survey (a pre-trial study) was conducted February 2012 (PCR prevalence 6.34%) [4, 25]. Four additional cross-sectional surveys were performed at the start of the rainy season in April/May (surveys 1, 2012 and 3, 2013) and six month later in October/November (surveys 2, 2012 and 4, 2013) with a PCR prevalence between 3 and 4% [25]. No differences in PCR prevalence were observed between the two study arms. PCR positive persons received a 3-day treatment with dihydroartemisinin plus piperaquine immediately following the national treatment guidelines [5, 25]. The immunoassay was performed on a total of 8,438 out of 26,929 blood spot samples. Of this total, 6,502 samples were randomly selected from survey 2 (3,264 out of 4,996 samples) and survey 4 (3,238 out of 5,431 samples). In addition, a total of 452 samples from the pre-trial and all four trial surveys were analysed as they were PCR positive for *Plasmodium* parasites. Furthermore, all samples of individuals that were randomly re-sampled three (430 individuals), four (36 individuals) or five times (10 individuals) during the entire MalaResT project were also included (Figure 1). For the immunoassay blood spot filter papers were prepared by punching one disc of 6mm (diameter), and eluted overnight in 160µL of PBS-TBN (PBS-1% BSA-0.15% Tween, pH 7.4, Sigma-Aldrich (dilution 1:40)). Just before use in the immunoassay, the eluted samples were diluted to 1:200 in PBS-CR, as previously described [26].

2.3 Antigens

The selection of Ags was based on their availability and tested in a previous study that focused on the implementation of the multiplex bead-based immunoassay [26]. The current assay includes eleven *Plasmodium* specific peptides, and eight *Plasmodium* specific recombinant proteins. These 19 Ags cover most stages of the life cycle of the parasite in the host (Table 1, Figure 1). In addition, two peptides specific for the *Anopheles gambiae* saliva protein gSG6 (salivary gland 6) were included. All peptides were chemically synthesized with an added N-terminal cysteine residue and BSA

(bovine serum albumin, Sigma-Aldrich, St. Louis, USA) [27] by GeneCust Europe (Dudelange, Luxembourg). The recombinant proteins were produced as described in Table 1.

Table 1 Overview of the antigens (peptides and recombinant proteins) used in this study.

Antigens	Sequence (N-terminal to C-terminal)	g/mol	Life-cycle stages	*Plasmodium species*	Peptide or recombinant protein
CSP	NANPNANPNANPNANPNVDPNVDPC	2557.67	Sporozoite	*P. falciparum*	Peptide
Pf13	C-terminal His-tag produced in *E.coli*		Sporozoite	*P. falciparum*	Recombinant
STARP.R	STDNNNTKTISTDNNNTKTIC	2299.42	Sporozoite & liver stage	*P. falciparum*	Peptide
SALSA1	SAEKKDEKEASEQGEESHKKENSQESAC	3123.24	Sporozoite & liver stage	*P. falciparum*	Peptide
SALSA2	NGKDDVKEEKKTNEKKDDGKTDKVQEKVLEKSPKC	4019.52	Sporozoite & liver stage	*P. falciparum*	Peptide
SR11.1	EEVVEELIEEVIPEELVLC	2213.5	Sporozoite & liver stage	*P. falciparum*	Peptide
LSA1.41	LAKEKLQEQQSDLEQERLAKEKLQEQQSDLEQERLAKEKEKLQC	5297.97	Liver stage	*P. falciparum*	Peptide
LSA1.J	ERRAKEKLQEQQSDLEQRKADTKKC	3046.43	Liver stage	*P. falciparum*	Peptide
LSA3.NR2	VLEESQVNDDIFNSLVKSVQQEQQHNVC	3230.53	Liver stage	*P. falciparum*	Peptide
LSA3.RE	VESVAPSVEESVAPSVEESVAENVEESVC	2991.2	Liver stage	*P. falciparum*	Peptide
Pf.MSP1.19	Glutathione S-transferase (GST) fusion protein. C-terminal expressed in *E.coli*		Merozoite	*P. falciparum*	
GLURP	EDKNEKGQHEIVEVEEILC	2241.47	Trophozoite	*P. falciparum*	Peptide
GLURP.P3	EPLEPFPTQIHKDYKC	1945.23	Trophozoite	*P. falciparum*	Peptide
Pf.GLURP.R2	C-terminal produced in *E.coli*		Trophozoite	*P. falciparum*	
Pvlike.CSP	APGANQEGGAAAPGANQEGGAAAPGANQEGGAAC	2892.99	Sporozoite	*P. vivax*	Peptide
PvVK210.CSP	DGQPAGDRAAGQPAGDRADGQPAGDRADGQPAGC	3206.3	Sporozoite	*P. vivax*	Peptide
PvVK247.CSP	ANGAGNQPGANGAGNQPGANGAGNQPGANGAGNC	2905.95	Sporozoite	*P. vivax*	Peptide
PvCSP chimera	Soluble His-tag protein expressed in wheat-germ cell free expression system		Sporozoite	*P. vivax*	Recombinant proteins
PvAMA1			Merozoite	*P. vivax*	Recombinant proteins
PvDBP			Merozoite	*P. vivax*	Recombinant proteins
Pv.MSP1.19	C-terminal produced in the baculovirus expression system		Merozoite	*P. vivax*	
PmCSP	GNAAGNAAGNDAGNAAGNAAGNAAGNAAGNAAC	2358.37	Sprozoite	*P. malariae*	Peptide
SALIV1	EKVWVDRDNVYCGHLDCTRVATFC	2830.22	Salivary gland proteins	*An. gambiae*	Peptide
SALIV2	ATFKGERFCTLCDTRHFCECKETREPLC	3324.84	Salivary gland proteins	*An. gambiae*	Peptide

Ags are organized according to the *Plasmodium* species and the life-cycle stages in the human host

2.4 Covalent coupling of antigens to the beads/microspheres

Each Ag was covalently coupled at a concentration of 4µg Ag/10^6 beads to 35x10^6 paramagnetic beads/beadset (MagPlex microspheres, Luminex Corp., Austin, TX, USA) [26]. BSA (Sigma-Aldrich, St Louis, USA) was coupled to an additional set of beads to serve as a background control. All beads and the BSA were mixed to prepare a microsphere working mixture. This mixture was then aliquoted and stored at 4°C in portions of 500µl per tube [26].

2.5 Bead-based immunoassay

The immunoassay was conducted as previously described [26]. First, a 500µl aliquot of the microsphere working mixture was diluted to a final volume of 5,000µl (1:10) with a concentration of 1,000 beads/Ag/well. From this microsphere working mixture 25µl was added in each well of a 96-well plate followed by 50µl of diluted serum sample (1:200) [26]. As a control for the immunoassay, a pool of negative sera (non-exposed European sera) was added to each plate in duplicate at a dilution of 1:100 in PBS-CR. A pool of positive sera containing sera from four individuals tested positive for *P. falciparum* and two for *P. vivax* [26] was added to each plate in duplicate at dilutions 1:100, 1:400 and 1:1600. For the washing steps PBS-TBN was used, and 100µl/well of secondary Ab (R-phycoerythrin$^+$-conjugated AffiniPure F(ab')$_2$ fragment of goat anti-human IgG, Jackson Immuno Research Laboratories) at a dilution of 1:500 was added [26]. All plates were analysed in a random order to minimize a bias. In a final step, beads were resuspended in 100µl of 5% PBS-BSA, pH 7.4. In total, 216 plates were analysed and read by the MAGPIX® system with a minimum amount of 400 beads per spectral address. Results were represented as the median fluorescent intensity (MFI-Additional file 1) [26].

2.6 Statistical analysis

A scheme of the data analysis can be found in Figure 1. Raw data were processed and analysed in R version 3.1.0. [28]. To assure the validity of the results of each plate a quality control was performed. Results were corrected for background signal by substracting the signal obtained with BSA-coupled beads (MFI_{BSA}) from the median value of the Ag-coupled beads (MFI_{Ag}), defined by $\Delta MFI = MFI_{Ag} - MFI_{BSA}$ [26]. The MFI-values of the high positive control pool samples and the percentage positivity ($\frac{\Delta MFI\ Low\ positive\ control\ (Ag1)}{\Delta MFI\ High\ positive\ control\ (Ag1)} * 100\%$) from the low positive control (50% value of the high positive control) pool samples were plotted in Levey Jenning Charts. The high

positive (100% value) is determined per Ag and based on the 1:100, 1:1400 and 1:1600 serum dilutions. The plates with samples outside the range of -2SD and +2SD were rejected and repeated (Additional file 2) [26].

Further differences between duplicate samples were explored using a quantile regression on an MA-plot using R package *'OutlierD'* [29]. M is the difference between the duplicate samples and A is the average of the duplicate samples [30, 31]. Samples that fell outside the lower 25% and upper 25% quantiles of this MA-plot were rejected (Additional file 3). Hereafter, the means of the duplicates were calculated per sample of each Ag. All analyses were carried out on the natural logarithm (ln) of the raw MFI in accordance with Helb *et al.* [13].

As age influences Ab-responses [11, 32], age categories were created based on the work of Kusi *et al.* [8]. To explore whether the age categories defined were al biologically relevant in the study region, the (ln)MFI values were plotted against age (Additional file 4). Moreover, to account for multiple comparisons Bonferroni corrections were applied to each of the following approaches [33, 34].

Towards selecting markers that detect contact with the parasite (recent of not recent), ln(MFI) values of PCR positive and PCR negative samples were compared per *Plasmodium* species. Generalized estimating equation (GEE) models were used (geeglm function in the R package '*geepack'* [35–37]), taking into account the within-cluster correlations that might exist due to the sampling design (village level clustering) [38]. The model also takes into account PCR positivity and age as factors allowing estimation of "population-averaged" effects [38, 39]. Different GEE-models were compared through ANOVA (a p-value < 0.05 was considered significant) tests to select the most appropriate model per Ag: (1) a null model (intercept-only model), (2) a model with only PCR prevalence as a dependent variable, (3) a model with only age groups as a dependent variable, (4) a model with PCR prevalence and age groups as dependent variables (without interaction and (5) with interaction).

To estimate the half-life of the Abs after effective treatment of malaria, the presence of Abs in the same individuals (642 persons) followed up over time (± 600 days) was examined [40]. Therefore, only individuals that were PCR positive in at least one survey were selected and the time of PCR positivity (and effective treatment) was taken as time point zero. This allows assessing the dynamics of the Abs against different Ags. Three linear models were compared per Ag through ANOVA tests: (1) a model with time since infection as dependent variable, (2) a model with time since

infection and age groups as dependent variable (without interaction and (3) with interaction). Based on the slope (=λ) of the selected model, the half-life was estimated per serological marker ($t_{½} = \frac{-\ln(2)}{\lambda}$) [8, 41]. The 95% confidence intervals (95% CI) were estimated using the *'confint'* function in R [28]. This analysis was performed on the systematically parasite negative individuals as well.

For determining the association between Ab-levels at population level and PCR-prevalence of asymptomatic *Plasmodium* infections, the ln(MFI) values were plotted against PCR prevalence at cluster level. Therefore, forest plots (multiplot function in the R package *'grid'* [28] and forest plot function in R package *'ggplot2'* [42]) were created on the cluster prevalence data from survey 2 and 4 per *Plasmodium* species. For *P. falciparum* and *P. vivax* this was performed on mono infections and for the *An. gambiae* saliva proteins on all infections (regardless of *Plasmodium* species). Each cluster was examined twice, once for survey 2 and once for survey 4. Then, the prevalence data were ordered from low to high prevalence (Additional file 5) and split into different groups representing low, medium and high prevalence (cut2 function in R package *'Hmisc'* [43]). For each species, groups of clusters that showed an overlap between medium and high-level PCR prevalence were excluded, allowing a clear distinction between three groups (high-, medium- and low-level PCR prevalence). Thereafter, ln(MFI) values were age-adjusted as differences in Ab-levels can be found between different age categories. Finally, linear models taking into account cluster within survey as random effect (lmer function in R package *'lme4'* [44]) were compared through ANOVA tests: (1) a null model (intercept-only model), (2) a model with PCR prevalence as a dependent variable, (3) a model with PCR prevalence and age as dependent variables (without interaction and (4) with interaction). The Incidence Rate Ratio (IRR) is corresponding to a ratio of Ab-levels estimated directly with the R-software.

3 Results

Although PmCSP was included in the battery of Ags, the results for PmCSP are not shown as the MFI signal (mean MFI < 600) did not surpass the background signal for the BSA.

3.1 Quality control

A total of 8,654 field blood samples were analysed distributed over 108 96-well plates. Each plate was analysed in duplicate. After quality control, out of the 216 plates 30

plates were repeated (Additional file 2). The control samples fell within the linear range of the assay (Additional file 6). Samples were checked for uniformity across the measuring range of the assay by looking at the between plate variation of the samples (> 96%). Thereafter, based on the quantile regression (Additional file 3), between 10% and 17% of the duplicate samples depending on the Ag were not consistent with each other and were excluded from the dataset. When determining the age categories, samples from individuals above 50 years were excluded, due to large variations in MFI values and a limited number of available samples (Additional file 4).

3.2 Comparison of MFI values between PCR positive and PCR negative samples

For 17 out of 20 Ags, the Ab levels were higher in PCR positive as compared to PCR negative blood samples ($p < 0.0025$, Bonferroni correction for 20 different tests) (Figure 2). However, Ags PvVK210.CSP ($p=0.842$), PvVK247.CSP ($p=0.557$) and PvCSP ($p=0.009$) did not show significant differences between the Ab levels within the age groups. As such, these three Ags were omitted from the figure. For 12 out of 20 Ags (CSP, Pf13, SALSA2, SR11.1, LSA1.J, LSA3.RE, Pf.MSP1.19, PvAMA1, PvDBP, Pv.MSP1.19, SALIV1 and SALIV2) there was no interaction between PCR status and age group, meaning that PCR-positive persons have significantly higher Ab levels for 12 Ags than PCR-negative persons (Figure 2), within all age groups (model 4, $p < 0.0038$).

For five other Ags the model that includes the interaction between PCR-positivity and the age groups was selected as the best model, showing that there is a significant difference in MFI values between PCR positive and PCR negative samples ($p<0.01$, Bonferroni correction for five tests), but that the magnitude of the difference depends on the age group. These Ags can be divided into two different groups. First PvEBP shows a significant difference ($p<0.01$) between MFI values of PCR positive and PCR negative persons only in the younger age groups (2-5 and 6-15 years old), whereas for STARP.R, LSA1.41, GLURP and Pf.GLURP.R2 the differences in MFI values between PCR-positive and PCR-negative persons are significant ($p<0.01$) in the older age groups (6-15 and 16-50 years old) only.

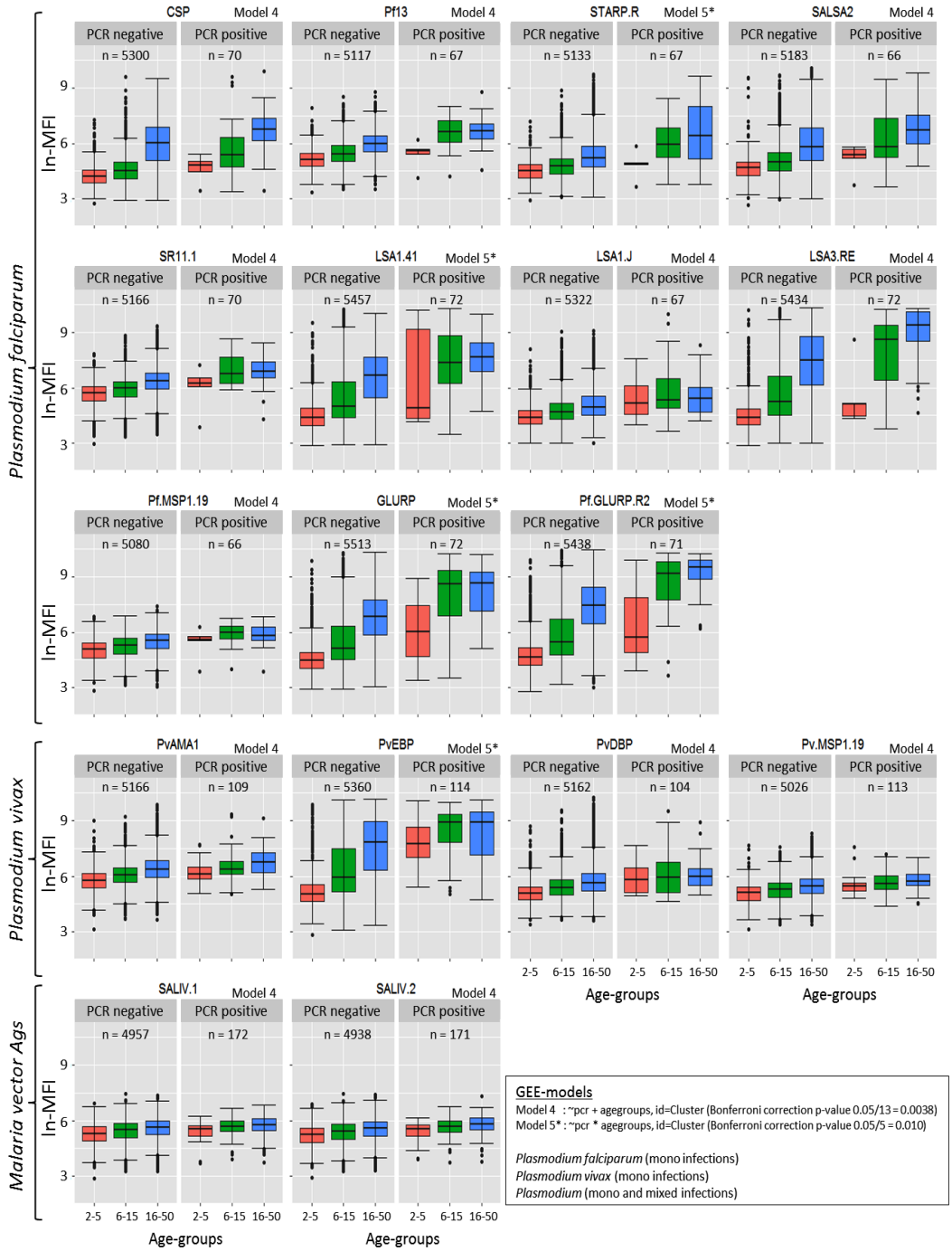

Figure 2: Antibody responses to *Plasmodium* antigens, stratified by age and the presence or absence of malaria infection. Individuals were divided into 3 age groups (1-5, 6-15 and 16-50) to explore the relation with age. Previous obtained PCR results [4, 5] were used to determine the presence and absence of the *Plasmodium* infection. Boxplots represent the medians, interquartile ranges and error bars show 95% confidence intervals. Circles represent outlier values. Generalized estimating equation (GEE) models were conducted taking into account Cluster as a random effect. Ags with non-significant results were not included in the figure.

3.3 Ab-responses decline with days since infection, depending on age and serological marker

There is a lot of variation between the estimated half-life per serological marker, suggesting that it is possible to distinguish short (seasonal) and long-term (year round) transmission trends. The model with time since infection and age groups as dependent variables (without interaction) was selected as the best model for all 20 Ags, suggesting that age does not have an effect on the slope of the Ab decay, although the Ab titres at time point zero differ between age groups (Figure 3; Figure 4). For nine antigens (CSP, STARP.R, SALSA2, SR11.1, LSA1.J, LSA3.RE, GLURP, Pf.GLURP.R2 and PvEBP) a pronounced decay of the Ab titres was observed (Figure 3). When comparing these graphs with the group of individuals being systematically negative, Ags LSA3.RE, GLURP and Pf.GLURP.R2 showed a much steeper decay in the group of individuals being PCR positive one time. Besides, the graphs of the PCR positive seems to flow into the negative groups, showing a difference between the intercept of >2. This outcome suggests that these Ags are most promising for reflecting short-term patterns in malaria transmission. Estimated half-lives, ranging between 6 months (176 [CI: 119 - 338] days) for Pf.GLURP.R2 and more than two years for some *P. vivax* Ags, clearly depend on the serological marker used (Figure 5, Additional file 7). Abs against five Ags (Pf.GLURP.R2, SR11.1, LSA3.RE, GLURP and Pf.MSP1.19) show estimated half-life values shorter than approximately 7.5 months, with estimated half-lives ranging from 6 months (176 [CI: 119 - 338] days) until ~7.5 months (225 [CI: 171 - 329] days). Estimated half-lives of Abs against STARP.R, Pf13, CSP, SALSA2 and SALIV.2 range between ~8.5 months (263 [CI: 159 - 768] days) until ~1 year (373 [CI: 284 - 546] days) and of Abs against LSA1.J, SALIV.1, LSA1.41, PvAMA1 and PvMSP1.19 from ~1.1 year (402 [CI: 203 - 16035] days) until ~1.6 year (597 [CI: 350 – 2020] days). Remaining Abs show an estimated half-life of more than 2 years.

For this analysis for some Ags (CSP, STARP.R, SALSA2, SR11.1, LSA1.41 and Pf.MSP1.19) samples from young children (2-5 years) were not included, due to a limited amount of samples available. For the same reason the age group of 16-50 years old was excluded for LSA3.RE.

3.4 Intensity of the Ab-responses decreases together with the malaria exposure

To test whether Abs detect recent malaria exposure in a population, the relation between Ab-titres (ln(MFI)) and the PCR prevalence of malaria in the province of Ratanakiri was explored. Therefore, the cluster PCR prevalence data from survey 2 and 4 was divided into different groups (forest plots: Additional file 5).

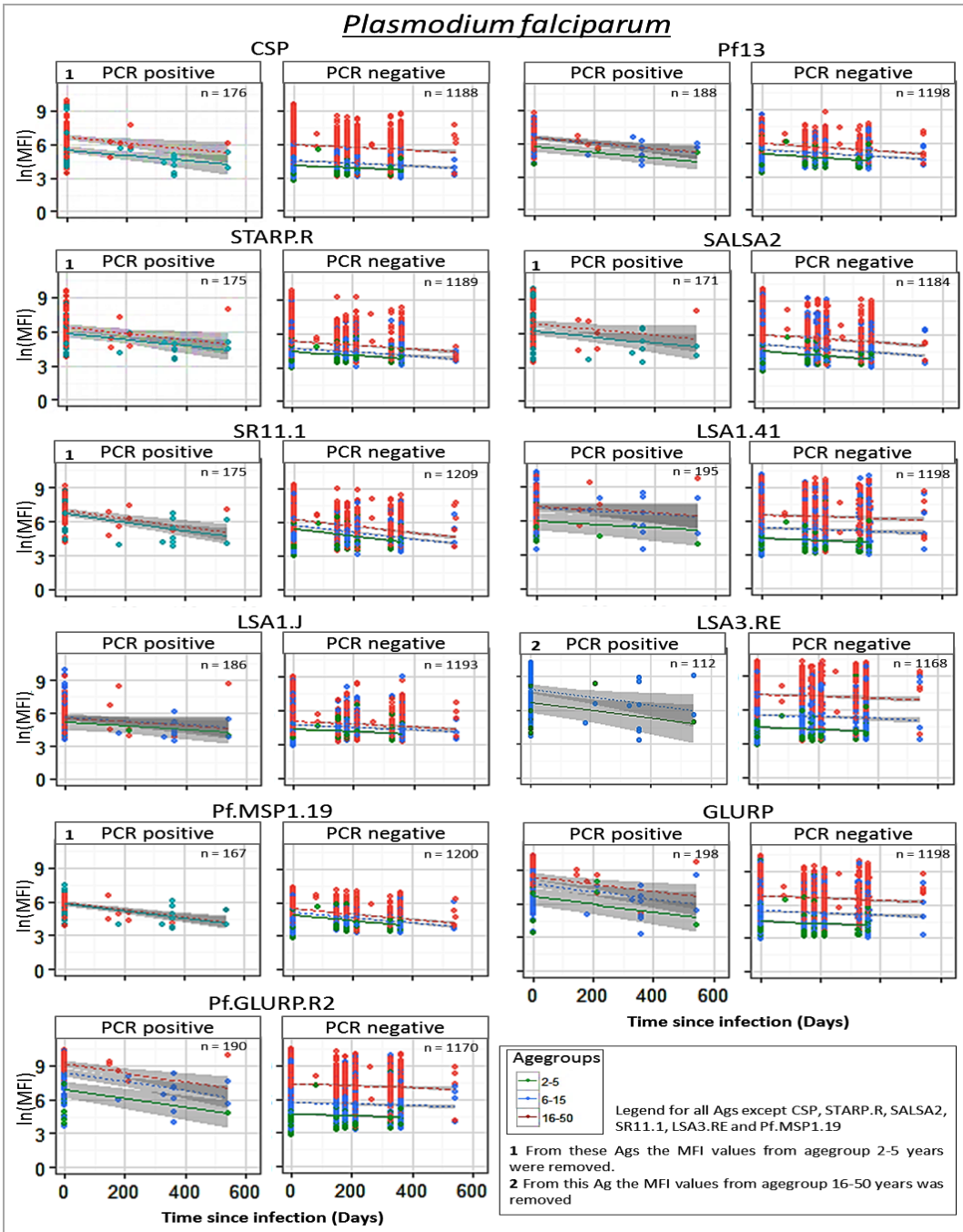

Figure 3: Plasmodium falciparum Ab profiles in relation to time since last infection compared to the systematically negative individuals. Each serological marker is analysed for Plasmodium infection and 3 age groups (1-5, 6-15 and 16-50). Samples collected through longitudinal follow-up were aligned, starting with putting the malaria episode detected by PCR at time point zero. Points represent the different samples in which a linear regression is drawn through with the 95% confidence intervals. These outcomes were then compared to the group of individuals being systematically negative over time, to see if the Ab-levels follow a similar or different decay over time between the parasite positive and negative individuals.

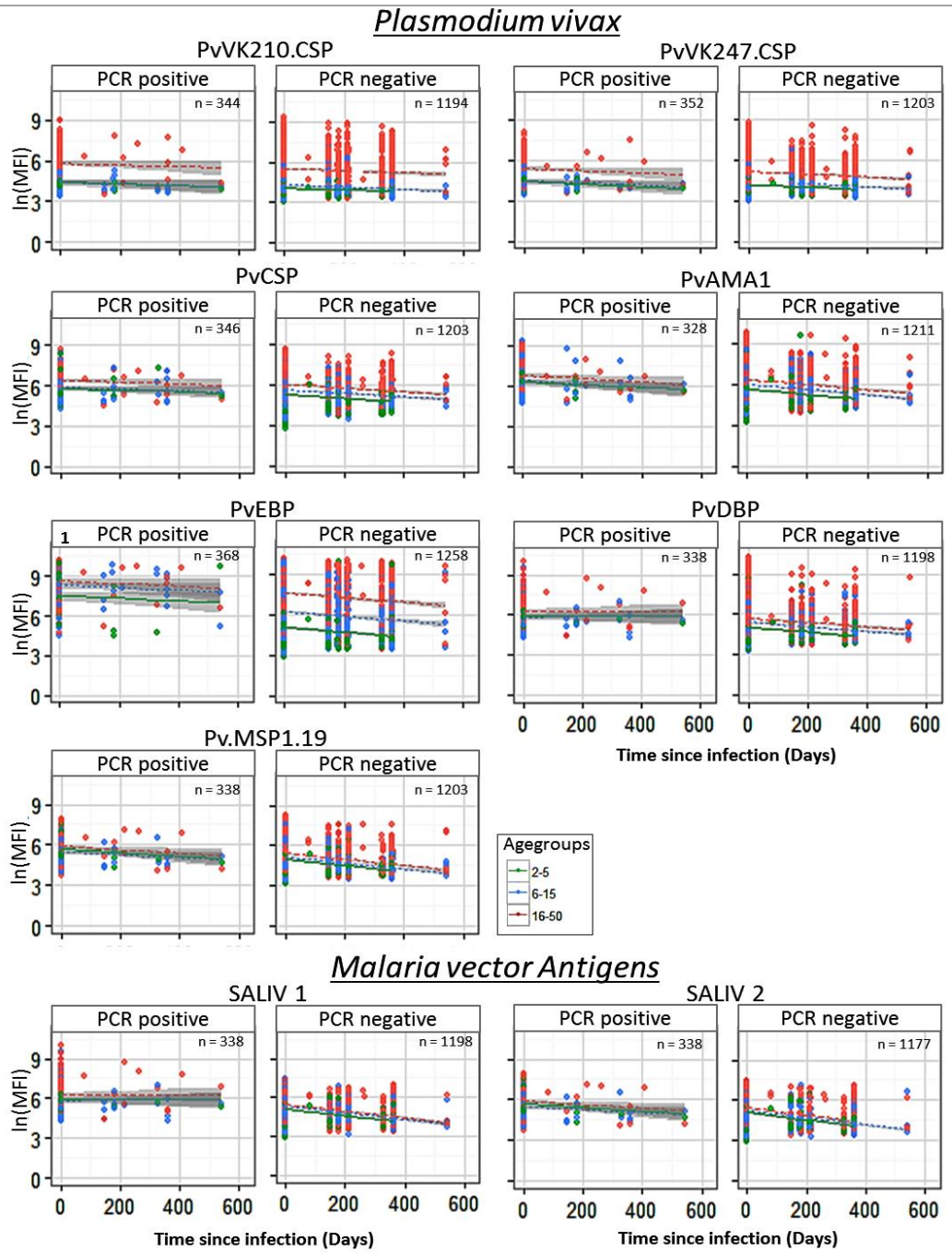

Figure 4: *Plasmodium vivax* Ab profiles in relation to time since last infection compares to the systematically negative individuals over time. Each serological marker is analysed for *Plasmodium vivax* infections and 3 age groups (1-5, 6-15 and 16-50). Samples collected through longitudinal follow-up were aligned, starting with putting the malaria episode detected by PCR at time point zero. Points represent the different samples in which a linear regression is drawn through with the 95% confidence intervals. These outcomes were then compared to the group of individuals being systematically negative over time, to see if the Ab-levels follow a similar or different decay over time between the parasite positive and negative individuals.

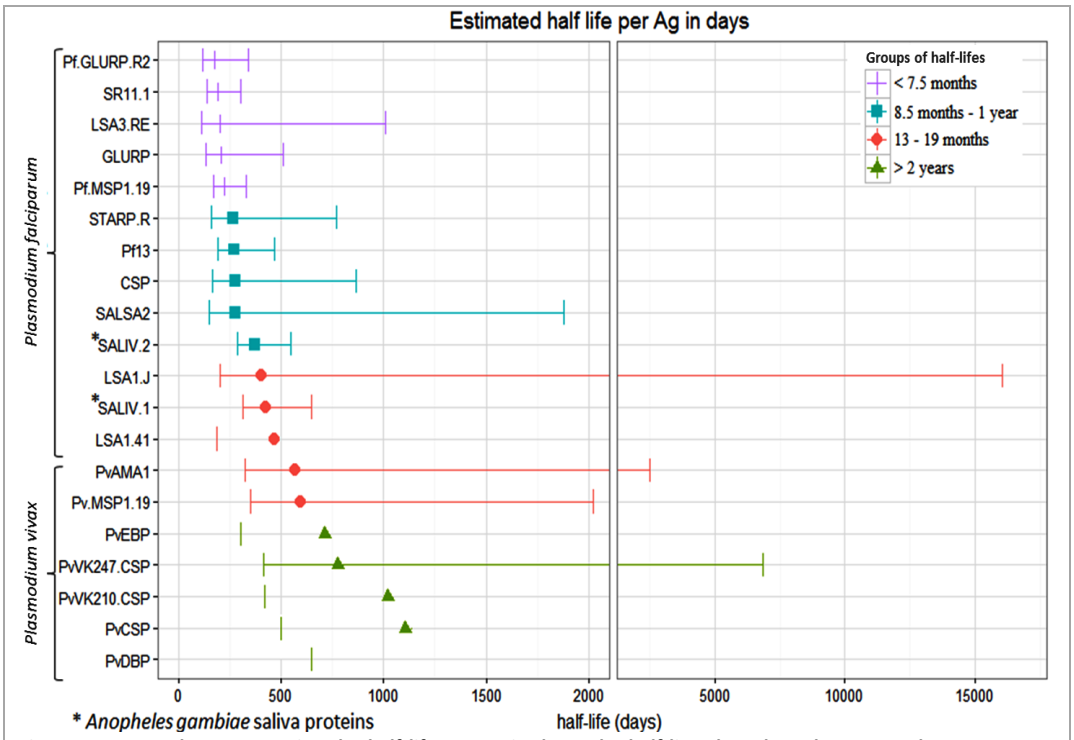

Figure 5: Forest plot representing the half-life per Ag in days. The half-lives based on the repeated measurement samples were estimated in days. A linear regression model was fitted on log-transformed MFI data taking into account age as factor. Estimated slopes and their 95% confidence intervals were used to obtain the half-life in days ($t_{½} = \frac{-\ln(2)}{\lambda}$). This forest plot represents the half-lives of each Ag ordered from the shortest to the longest half-life. The purple crosses represent an estimated half-life shorter than 7.5 months, the blue squares range from 8.5 months until one year, the red dots range from 13 until 19 months, and the green triangles represent an estimated half-life longer than 2 years. The error bars are the 95% confidence intervals. A remarkable founding in this figure is that *P. falciparum* shows a clear difference between the short and long lived Ab-responses, while for the *P. vivax* only long lived Ab-responses are seen.

In the forest plot, clusters with PCR prevalence between 3.5 percent and 7 percent were situated in the overlap between the medium- and high-level PCR prevalence and were not considered for present analysis, reflecting only those exhibiting high (7 - >10%), medium (1 - 3.5%), and low (0 - <1%) PCR-prevalence levels (Table 2). For Ags SALIV.1 and SALIV.2 all *Plasmodium* data (mono and mixed infections) were used, whereas the *P. falciparum* and *P. vivax* Ags were analysed species specific (only mono infections).

For all 20 Ags, the model with PCR prevalence levels and age groups as dependent variables (without interaction) was selected. This model shows that when the PCR prevalence in groups of villages becomes lower, MFI levels also decrease ($p < 0.0025$; Additional file 8), within all age groups. Moreover, with decreasing endemicity Ab responses to the Ags presented in Figure 6 show a more marked decline (drop in IRR

between PCR prevalence level in respect to the reference group), as well as an increase in IRR with age. Biggest decline in IRR between PCR prevalence level was seen for Ags LSA3.RE, GLURP, Pf.GLURP.R2 and PvEBP, whereas the highest increase in IRR with respect to age was seen for Ags CSP, LSA1.41, LSA3.RE, GLURP, Pf.GLURP.R2 and PvEBP compared to the other Ags (Figure 6).

3.5 Selection of informative antigens for recent infection are based on three criteria

To determine which Ags best reflect recent malaria exposure (seasonal) the following three criteria were used: (1) the outcome of the estimated Ab-responses based on the differences in ln(MFI) between PCR positive and PCR negative individuals, (2) the assessed half-lives and (3) the estimated Ab-responses in relation to high-, medium- and low levels of *Plasmodium* exposure (Table 3). Based on these criteria the Ags that are most likely to reflect recent malaria exposure to *P. falciparum* are LSA3.RE, GLURP and Pf.GLURP.R2. Among the seven Ags tested for *P. vivax*, PvEBP is the most prominent choice.

Table 2 Amount of cluster communities selected per species and per PCR prevalence level. Cluster communities were ordered per survey exhibiting high-, medium- and low-levels of PCR prevalence. This was performed on PCR data from all *Plasmodium* species (mono and mixed infections), *P. falciparum* (mono infections) and *P. vivax* (mono infections).

PCR prevalence		*P. falciparum*	*P.vivax*	*Plasmodium*
High	S2	13	14	19
	S4	3	9	10
Medium	S2	18	26	24
	S4	21	23	24
Low	S2	52	39	26
	S4	61	44	31

Table 3 Three criteria to select the most promising antigens. Three criteria are used to select the most promising Ags for recent malaria infection. These criteria are based on: (1) the outcome of the estimated Ab-responses based on the differences in ln(MFI) between PCR positive and PCR negative individuals, (2) the assessed half-lives with the best Ab-responses of < 7.5 months followed by Ab-responses between 8.5 months until 1 year and finally (3) the sensitivity to the level of malaria endemicity in communities.

		Ags selection based on the three criteria			
	Antigens	**PCR^+ vs PCR^- values [(A)]**	**Half-lives (PCR^+) [(B)]**	**Differences in Intercept [(C)]**	**sensitivity by endemicity [(D)]**
P. falciparum	CSP	+	+	+	+
	Pf13	+	+	-	+
	STARP.R	++	+	+	+
	SALSA2	+	++	+	+
	SR11.1	+	+	+	+
	LSA1.41	++	+	-	++
	LSA1.J	+	+	+	+/-
	LSA3.RE	++	++	++	++
	Pf.MSP1.19	+	-	-	-
	GLURP	++	++	++	++
	Pf.GLURP.R2	++	++	++	++
P. vivax	PvVK210.CSP	-	-	-	+/-
	PvVK247.CSP	-	-	-	+/-
	PvCSP	-	-	-	-
	PvAMA1	-	-	-	+/-
	PvEBP	++	-	++	++
	PvDBP	-	-	-	-
	Pv.MSP1.19	-	-	-	-
Vectors	SALIV1	-	-	-	-
	SALIV2	-	+	-	-

(A) Difference in ln(MFI) (ln-MFI PCR+ - ln-MFI PCR-); ++ (value > 1), + (value > 0.5), - (value < 0.5)

(B) Half-lives estimated via linear regression models; ++ (half-life < 7.5 months), + (half-life between 8.5 months - 1 year), - (half-life > 1 year)

(C) Difference in intercept between PCR+ and PCR- for half-life estimation; ++ (intercept difference > 2), + (intercept difference between 1 - 2), - (intercept difference <1)

(D) Sensitivity by endemicity estimated via linear regression models; ++ (IRR of PCR prev. decline with > 0.2, IRR increase by age with > 4), + (IRR of PCR prev. decline with > 0.1, IRR increase by age with > 1), -

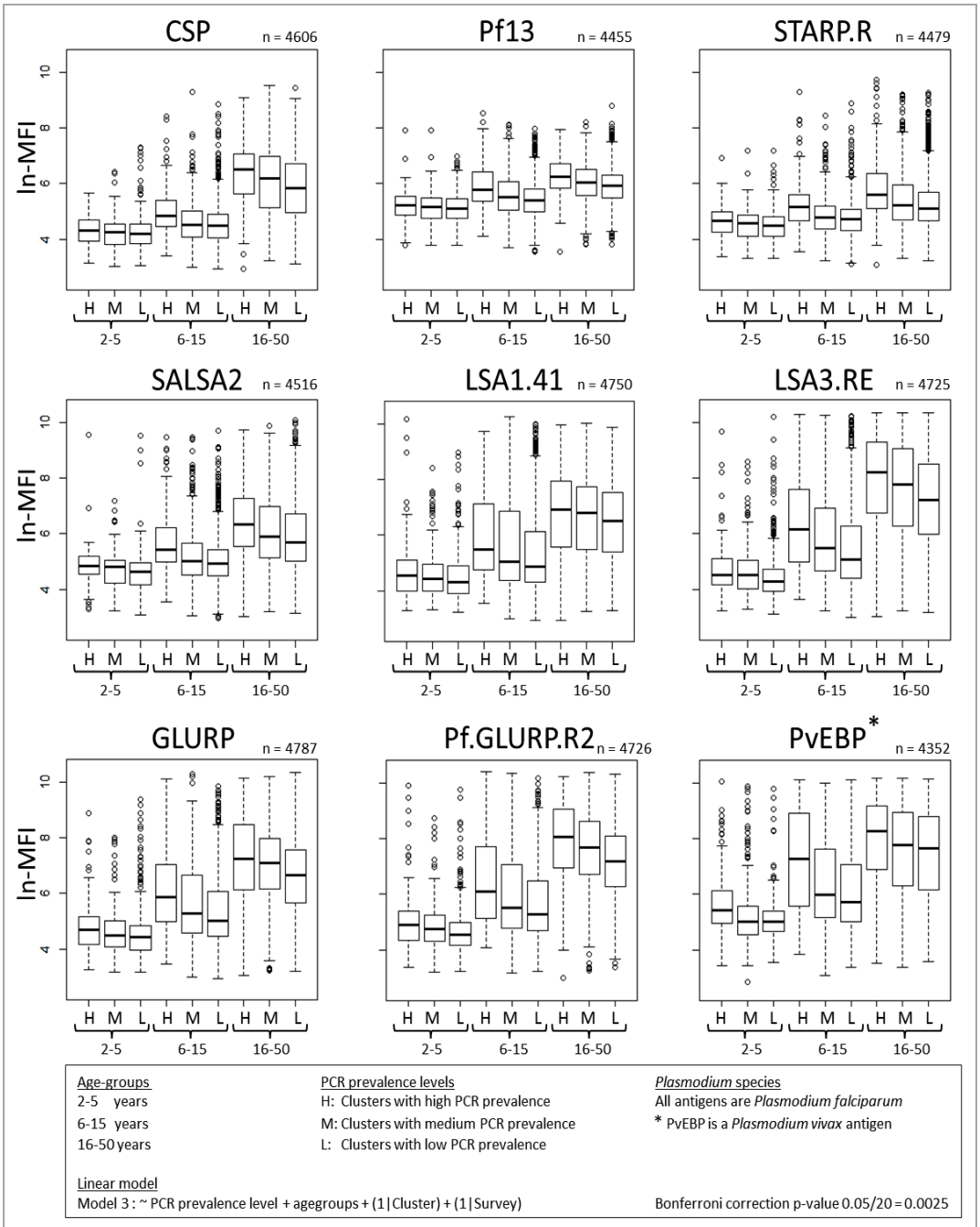

Figure 4: Ab-responses to *Plasmodium* Ags in relation to the risk of malaria exposure in different villages. To examine the associations between the Ab-levels and the malaria exposure clusters of villages were divided into three groups (those with high- (7 - >10%), medium- (1 - 3.5%), and low- (0 - <1%) prevalence levels). In addition, because age plays a major role in the analysis of Ab-responses, the three age groups (2-5, 6-15 and 16-50 years old) were included. Boxplots represent the medians, interquartile ranges and error bars show 95% confidence intervals. Circles represent outlier values. Ags showed to be statistical significant for model 3, a model with three groups of villages and age groups as a dependent variable (without interaction) taking into account Cluster and Survey as random effects. The figure only represents the Ags with the steepest decay (slope < -0.6).

4 Discussion

Several research groups recently developed multiplex serological assays for the detection of Abs against *Plasmodium* Ags [27, 38, 45–48]. Previous studies combining short- and long-lived markers for *Plasmodium* have generated knowledge about past and recent changes in malaria transmission within communities [13, 40, 49]. This has led to an increasing interest in the use of serology in acquiring epidemiological information that can be implemented in malaria control programmes [9, 13, 40, 49–55]. At this point, the majority of the studies and elimination programmes have focused in first instance on *P. falciparum* [13, 27, 38, 45, 46, 52, 56], which is easier to eliminate than *P. vivax* due to possible relapses in the latter one [57]. However, in contrast to other studies the current study was carried out in Southeast Asia (Cambodia) where all human malaria parasites are co-occurring [26, 58] and in which occupational and behavioural factors define the risk for malaria exposure [59–61]. As such, adolescents and mainly adults performing plantation work and forest activities are the main risk groups for malaria infection [59–62]. In this setting, insights into malaria transmission will be greatly enhanced by analysing age-adjusted Ab-responses directed against *Plasmodium* parasites including *P. vivax*. However, in this study age did not show any interactions on the outcomes.

The present study is to our knowledge the first study in Southeast Asia that uses multiplex serological measurements to document the half-life of IgG-Abs. Previous studies declare that Ab-levels follow a predictable pattern, in which every few months the Ab-level drops by half in the absence of re-infection [18, 41, 63]. To estimate this drop and apply those values in assessing the force of infection (FOI) in a study population, most researchers use a reversible catalytic conversion model [6, 40, 49, 51] on cross-sectional data. Such model is a simplification of a complex immunological process in which serological responses are converted to binary outcomes (seropositive or seronegative) through a threshold model [52]. Hence, these binary outcomes may lead to a loss of information due to the high range of MFI values within the seropositive group, and misclassification might occur because of subtle changes in the Ab-responses over time due to other infections or small laboratory variations [9]. Another limitation is that this model assumes a fixed sero-reversion rate, independent of age and transmission rate [51]. Therefore, some studies elaborated this model by comparing longitudinal- and cross-sectional data, which has led to large discrepancies in seroconversion and seroreversion rates [52]. Alternatively, linear regression models are used to provide information about the antibody acquisition [13, 21]. Helb *et al.* [13] and Yman *et al.* [12] estimated recent *P. falciparum* exposure at individual (longitudinal data) and population level (cross-sectional data). Studies based on

longitudinal data mainly use approaches such as linear regression models [13, 20], generalized linear models (GLM) [64], or Ab-intensity models [12] to analyse Ab profiles.

A first criteria for selecting serological markers indicative for recent infection, is that the marker can at least detect current infection. Therefore, the Ab intensity in *Plasmodium* infected people was compared to non-infected people. In agreement with results of previous studies, the present study shows that most Ab-responses (17/20) within each age group rise similarly by an infection. In contrast, in infected individuals Ab-responses to STARP.R, LSA1.41, GLURP, Pf.GLURP.R2 (among persons between 6 and 50 years) and PvEBP (among children from 2-15 years) seem to be influenced by age. [65]. The differences among children can be explained based on the role of immunological maturity-status [6], as children that acquire a malaria infection have the ability to boost their IgG titres, after which these Ab-titres decay again [66]. In this analysis it is important to take into consideration that the group of PCR-negatives is very large, and that PCR negative persons might have been infected in the six months between sampling periods. Moreover, for the PCR positive individuals, it is unknown when the infection was acquired.

In a next step, antibody decay rates (or half-lives) were estimated by using linear regression models that were fit on Ab-responses obtained from a cohort at different time points. In summary, the obtained results showed that the used serological markers could be divided in four groups with a wide variety of half-life estimates (Figure 1, Table 3). Even though, limited information on the Abs persistence is available, some studies have previously estimated Ab half-lives [18, 20]. A study performed by Drakeley *et al.* [40] found a very long half-life of 49.8 years for Abs against Pv.MSP1.19, and Ondigo *et al.* [67] found half-lives from < 1year (3 Ags) to moderate (5-20 years for 3 Ags) and very long (> 40 years for 5 Ags) by means of the threshold approach. Wipasa *et al.* [18] and Fowkes *et al.* [20] found a clearance of approximately 7.6 years [18] and 0.8~7.6 years [20], respectively, through linear regression models for the same Abs. The estimated Ab-clearance against merozoite Ags in the present study are best comparable with the latter results. However, these studies [18, 20] showed large ranges in 95% confidence intervals, which was also seen in the present study. The half-life estimates are in contrast with the underlying immunology showing the short period that merozoites need to reinvade erythrocytes. This is the short timespan in which the Abs have direct contact with the merozoite surface proteins, as this is the moment they are actually visible in the blood circulation [68]. A short clearance of Abs against merozoite Ags was also shown by Kinyanjui *et al.*

[41] who estimated a clearance of 6~10 days and White *et al.* [66] that found a half-life of 7-72 days in children and 3-9 years for IgG Abs against AMA1, MSP1, MSP2 and CSP in infants, with a linear regression models. All these different outcomes suggest that using different model approaches (reversible catalytic conversion model and linear regression model) provide different outcomes, whereas with the linear regression models shorter half-lives were obtained. Moreover, large discrepancies in 95% confidence intervals were seen for some sporozoite related Ags as well. For these serological markers lower MFI values were observed [data not shown], resulting in larger 95% confidence intervals of Ab half-lives. This is not surprising as small numbers of sporozoites are only briefly present in the blood circulation to stimulate a sufficient production of sporozoite Abs, particularly, when situated in a region with low malaria transmission as the Mekong Subregion [17, 19]. Therefore, it has been previously suggested that the use of serological markers based on the sporozoite stage of malaria parasites might lead to an underestimation of the malaria endemicity in low transmission areas, while they possibly are more sensitive in hyper- and holo-endemic settings [19].

Finally, the cross-sectional data were examined to investigate whether the serological markers were capable of picking up current differences in malaria prevalence. Overall, for a lower PCR prevalence in a group of villages, the observed MFI levels were also lower within all age groups. This trend was similar as what was observed by Ambrosino *et al.* [27], who observed a rise in malaria specific Ab-responses with an increasing malaria exposure per village [27].

Based on the three different criteria i.e. (1) sensitive to infection in individuals, (2) sensitive to the level of endemicity in communities, and (3) a short half-life of IgG-Abs, the best serological markers (LSA3.RE, GLURP and Pf.GLURP.R2) that reflect recent *P. falciparum* exposure were identified. Moreover, Ags, CSP, Pf13, STARP.R, SALSA2, SR11.1, LSA1.41 and LSA1.J also seemed to respond well to a lesser extent. Therefore, these Ags should not be ruled out for future research in different settings. PvEBP was selected as the better candidate for *P. vivax* surveillance.

5 Conclusion

Given the broad utility of serology, identifying serological markers for recent exposure seems a worthwhile investment [13]. In summary, it appears that for *P. falciparum* LSA3.RE, GLURP and Pf.GLURP.R2 are most likely to be reflective for recent exposure, whereas PvEBP is the only Ag from *P. vivax* that responds reasonably well, in spite of a half-life of more than one year. Therefore, it is essential to explore other Ags from *P.*

vivax. The only available *P. malariae* antigen did not provide a good response. It is remarkable that the best reflective Ags are mainly liver- and blood stage Ags, whereas in contrast to high endemic areas sporozoite level Ags are not useful in this setting (low-endemic). It is worth noting that all 20 Ags exhibit a direct link with the endemicity. In short, these Ags should certainly not be excluded for further in-depth analyses related to malaria control programmes. Another important aspect is that the use of Ab intensity data rather than dichotomizing the continuous Ab-titre data (positive *vs* negative) will lead to an improved approach for serological surveillance.

Declarations

Ethics approval and consent to participate

The study protocol was reviewed and approved by the Cambodian National Ethics Committee on Health Research (Approval 265 NECHR), the Institutional Review Board of the Institute of Tropical Medicine Antwerp (Approval IRB/AB/ac/154) and the Ethics Committee of the University of Antwerp (Approval B300201112714). Gatekeepers provided informed written consent for the participation of their village. The survey participant or his/her parents or guardian provided informed written consent for individual participation.

Consent for publication

Not applicable

Availability of data and material

The datasets supporting the conclusions of this paper are included within the paper. Raw data may be obtained from the corresponding author on request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Sample collection of the survey was performed by the CNM team (National Centre for Parasitology, Entomology and Malaria Control), the ITM Antwerp team and the Institut Pasteur du Cambodge team in Ratanakiri Province in Cambodia. The study design and literature research has been performed at the ITM by KK, LD and MC. Protocols were written by KK, based on protocols from Institut Pasteur de Madagascar (IVW), verified by LC and validated by DM. Screening of the blood spot samples was performed by KK, LW and SB. Selection of the pool of control sera and the high positive control dilutions was done by KK, LD and LC. Data entry and quality control was performed at the ITM by KK, LD, VS and LW. Analysis of the results was performed at the ITM by KK and VS and verified by LD and MC. The first draft of the manuscript was written by KK. All authors read and approved the final manuscript.

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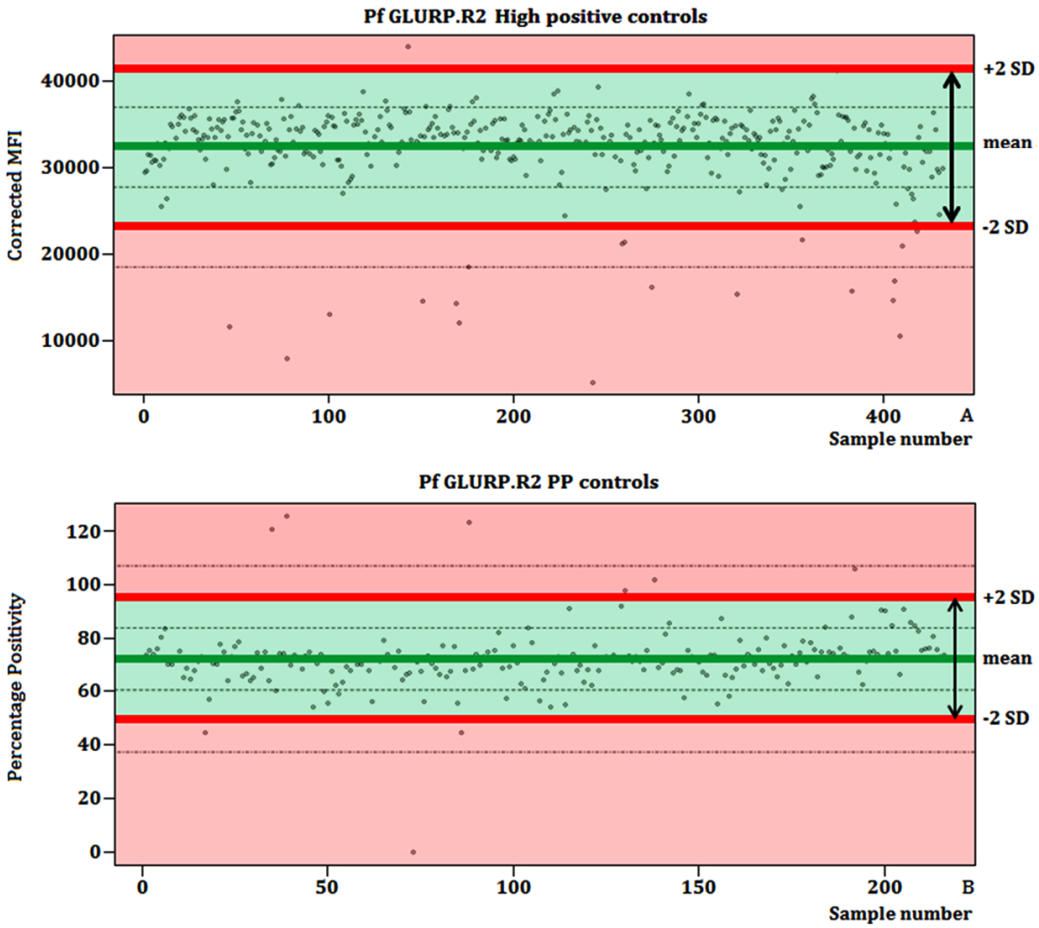
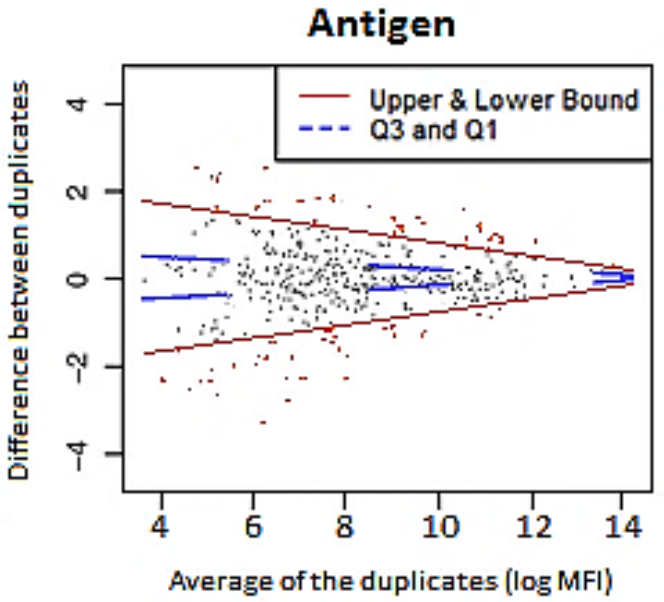
7 Additional files

Additional file 1 Raw MFI-data file. This file contains all raw data used for all analyses in this study.

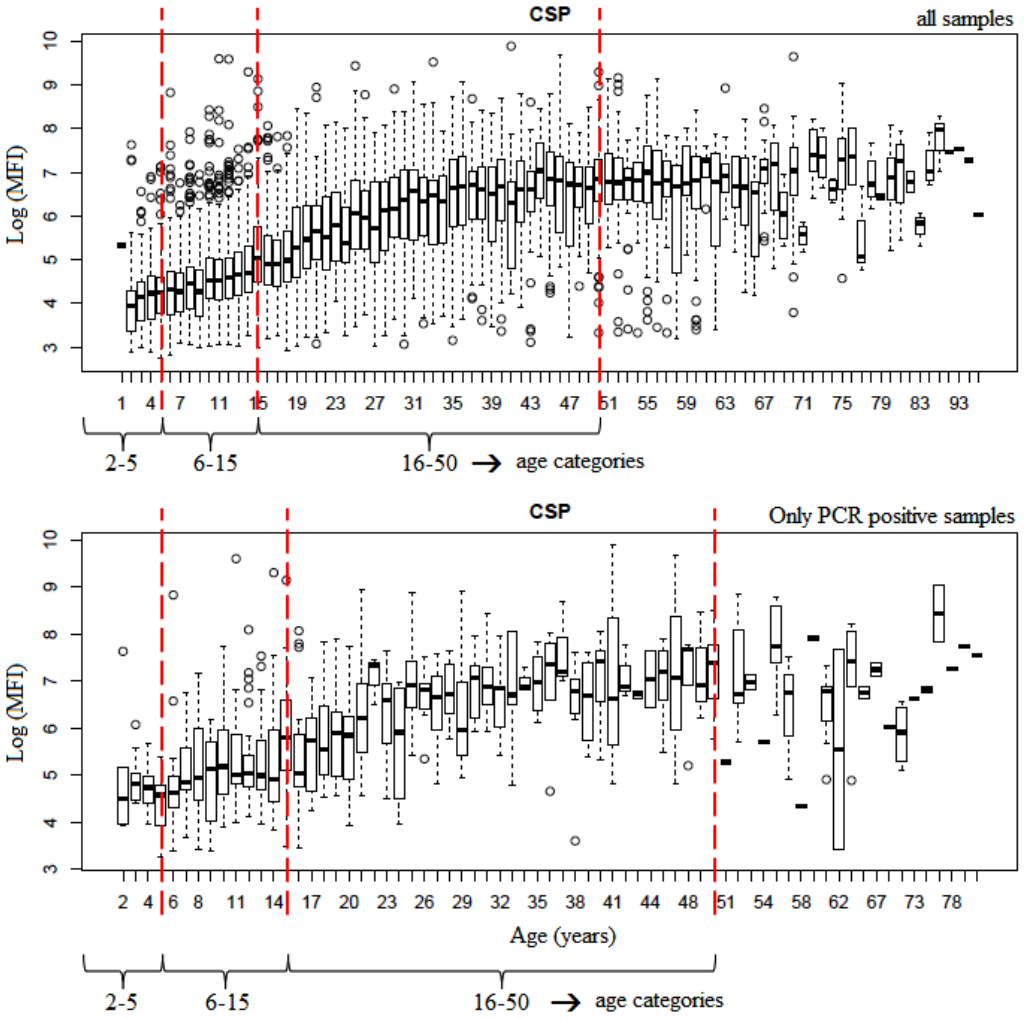
Additional file 2 Example of Levey Jenning Charts plotted for the quality control of the immunoassay used for screening the field bloodspot samples. Data analysis started with a quality control on the ΔMFI-values of the 100% positive control pool samples (A). The dots represent each positive control sera sample in duplicate per plate. If these dots fell out of the -2SD and +2SD (red area), these plates were rejected and re-analysed. The same quality control was also performed on the percentage positivity ($\frac{\Delta MFI\ Low\ positive\ control\ (Ag1)}{\Delta MFI\ High\ positive\ control\ (Ag1)}$*100%*) calculated from the 50% positive control pool samples per Ag (B). Based on the outcome of both graphs, 30 plates were rejected and reanalysed.



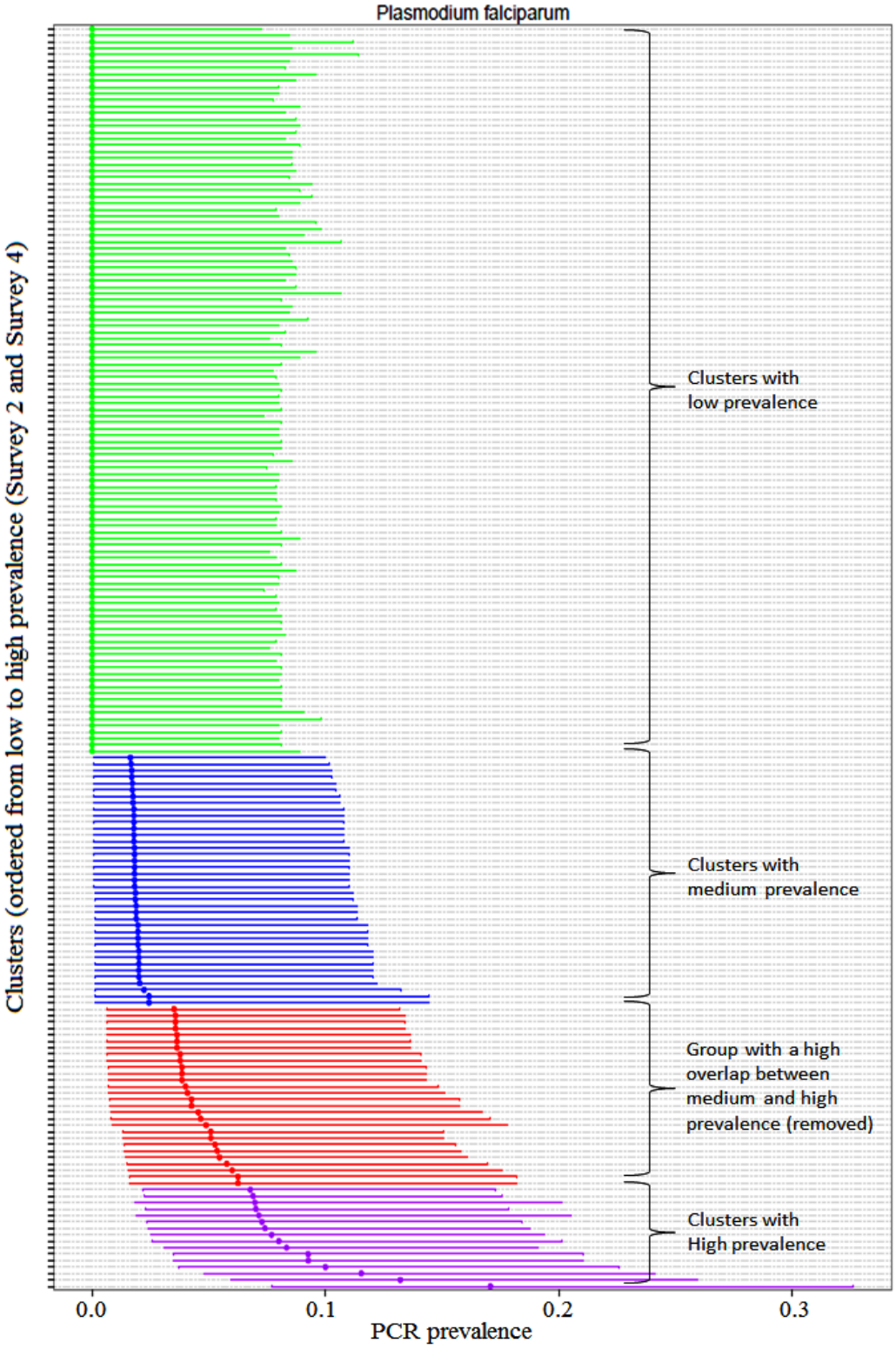
Additional file 3 Example of quantile regression model utilized on a MA-plot to detect differences between the duplicate samples. The quantile regression model is a way to validate samples and their duplicates on consistency. The red and black dots represent the samples and duplicates of which the red dots represent the outliers. The dotted lines represent upper and lower fences (Q3 + 1.5IQR and Q1 – 1.5IQR), where Q1 is the lower 25th quantile and Q3 the upper 25th quantile and IQR = Q3 – Q1. The outer solid lines represent lower and upper bounds that classify the difference between the outliers and non-outliers.



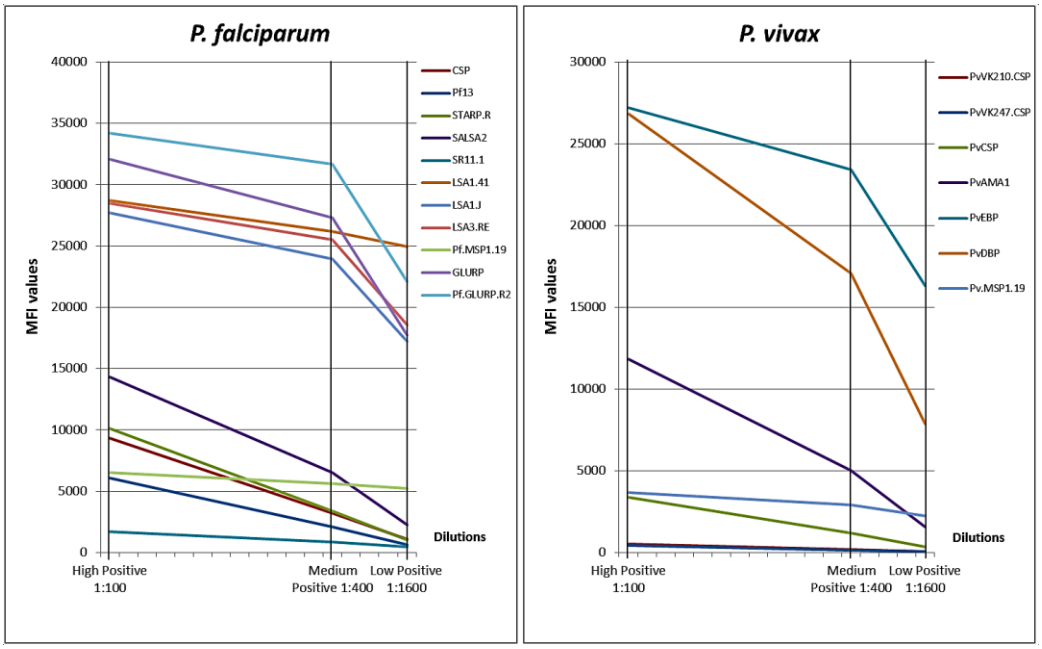
Additional file 4 Example of a histogram in which the MFI values are plotted against different age categories. Given the trend between the various ages it has been observed that this might be an important factor that has to be taken into account. The boxplots represent the medians, interquartile ranges and error bars (95% confidence intervals) per age. Circles represent outlier values. In the first plot is performed on all 8,654 samples, while the second plot has been performed on only the PCR positive samples. Looking at the trend it is clear that the MFI increase according to the age. Samples above the age of 50 show variation in MFI levels and were therefore removed from the analysis. Therefore age groups from 2-5, 6-15 and 16-50 years are chosen.



Additional file 5 Example of a Forest plot created to define the different levels of Clusters according to the PCR prevalence. To analyse Abs that detect recent changes in malaria transmission, forest plots were created on the cluster prevalence data from survey 2 and 4 per *Plasmodium* species (mono infections). Each cluster was examined twice, once for survey 2 and once for survey 4. The dots represent the measured PCR prevalence (% proportion) for a certain village. The error bars represent the 95% confidence intervals. The villages are ordered from the village with the lowest to the village with the highest PCR % proportion. Different groups are automatically selected with a R-software [32] package. The group that showed a high overlap between two different levels of prevalence was removed for further analysis.


Plasmodium falciparum
Clusters (ordered from low to high prevalence (Survey 2 and Survey 4)
Clusters with low prevalence
Clusters with medium prevalence
Group with a high overlap between medium and high prevalence (removed)
Clusters with High prevalence
0.0
0.1
0.2
0.3
PCR prevalence

Additional file 6 Multiplex assay on a dilution series of the control samples to confirm the linear range of the assay. The mean MFI values of the control samples (per dilution and per Ag) were plotted. Graphs were made species specific. The vertical black lines show the spot where the 1:100, 1:400 and 1:1600 dilution is situated. Each of the Ags follow the linear range of the assay.



Additional file 7 Estimates of the half-life per serological marker. The half-lives based on the repeated measurement samples were estimated in days. A linear regression model was fitted on log-transformed MFI data taking into account age as factor. Estimated slopes and their 95% confidence intervals were used to obtain the half-life in days ($t_{½} = \frac{-\ln(2)}{\lambda}$).

		Once PCR positive			Systematically PCR negative			Difference in intercept between PCR+ and PCR-
	Antigens	Half-life (days)	Lower CI	Upper CI	Half-life (days)	Lower CI	Upper CI	
P. falciparum	CSP	277	165	866	569	468	726	1.39
	Pf13	271	191	465	430	384	490	0.77
	STARP.R	263	159	768	401	352	467	1.4
	SALSA2	277	149	1881	735	321	450	1.61
	SR11.1	190	139	303	231	213	251	1.27
	LSA1.41	465	191	NA	857	588	NA	0.83
	LSA1.J	402	203	16035	571	481	701	1.5
	LSA3.RE	199	110	1009	785	539	1443	2.24
	Pf.MSP1.19	225	171	329	297	275	324	0.95
	GLURP	207	130	512	704	520	1089	2.09
	Pf.GLURP.R2	176	119	338	829	582	1441	2.18
P. vivax	PvVK210.CSP	1012	415	NA	885	676	NA	0.34
	PvVK247.CSP	777	412	6855	725	602	910	0.24
	PvCSP	1095	499	NA	508	446	NA	0.54
	PvAMA1	570	322	2515	367	328	415	0.74
	PvEBP	734	296	NA	386	316	NA	2.44
	PvDBP	NA	645	NA	NA	363	NA	0.85
	Pv.MSP1.19	597	350	2020	312	287	341	0.7
Vectors	SALIV.1	424	315	647	273	253	296	0.47
	SALIV.2	373	284	546	253	236	273	0.48

Additional file 8 Incidence rate ratios and p-values estimated to indicate difference between the Ab intensity (ln(MFI)) and the levels of exposure per age group (6-15 and 16-50 years). Significant differences between the different levels of exposure (High-, Medium-, Low prevalence) were seen for all specific Ags ($p<0.00025$). The incidence rate ratios (IRR) confirm that the MFI value decline together with the PCR prevalence in the villages and rise with age. Incidence Rate Ratio that indicates for how much (if > 1) or less (if < 1) the covariates decline with PCR prevalence level or rise with an increasing age. This is performed in respect to the reference category and LCI and UCI representing the lower and upper 95% confidence intervals based on the total sample size of n = 6,499 individuals from 98 villages.

		Plasmodium falciparum			Plasmodium vivax			Anopheles gambiae salivary proteins		
Species	Variables	level	IRR [LCI - UCI]	p-value	Species	IRR [LCI - UCI]	p-value	Species	IRR [LCI - UCI]	p-value
CSP	PCR prev.	High	reference	< 0.0025	PvVK210.CSP	reference	< 0.0025	SALIV.1	reference	< 0.0025
		Medium	0.784 [0.629 - 0.977]			0.851 [0.706 - 1.026]			0.870 [0.646 - 1.172]	
		Low	0.685 [0.562 - 0.835]			0.809 [0.683 - 0.957]			0.804 [0.626 - 1.051]	
	Age	2-5	reference			reference			reference	
		6-15	1.444 [1.325 - 1.574]			1.208 [1.118 - 1.307]			1.214 [1.155 - 1.276]	
		16-50	5.547 [5.115 - 6.016]			3.705 [3.442 - 3.988]			1.359 [1.296 - 1.425]	
Pf13	PCR prev.	High	reference	< 0.0025	PvVK247.CSP	reference	< 0.0025	SALIV.2	reference	< 0.0025
		Medium	0.812 [0.653 - 1.009]			0.812 [0.691 - 0.953]			0.875 [0.644 - 1.187]	
		Low	0.710 [0.584 - 0.864]			0.768 [0.664 - 0.887]			0.802 [0.609 - 1.056]	
	Age	2-5	reference			reference			reference	
		6-15	1.473 [1.390 - 1.562]			1.229 [1.154 - 1.308]			1.224 [1.165 - 1.287]	
		16-50	2.370 [2.243 - 2.504]			2.433 [2.294 - 2.581]			1.385 [1.322 - 1.452]	
STARP.R	PCR prev.	High	reference	< 0.0025	PvCSP	reference	< 0.0025			
		Medium	0.702 [0.552 - 0.892]			0.887 [0.746 - 1.056]				
		Low	0.622 [0.501 - 0.773]			0.796 [0.680 - 0.930]				
	Age	2-5	reference			reference				
		6-15	1.384 [1.283 - 1.493]			1.439 [1.360 - 1.523]				
		16-50	2.386 [2.222 - 2.563]			2.048 [1.942 - 2.160]				
SALSA2	PCR prev.	High	reference	< 0.0025	PvAMA1	reference	< 0.0025			
		Medium	0.671 [0.511 - 0.881]			0.933 [0.754 - 1.154]				
		Low	0.576 [0.451 - 0.736			0.774 [0.639 - 1.507]				
	Age	2-5	reference			reference				
		6-15	1.650 [1.493 - 1.823]			1.413 [1.324 - 1.507]				
		16-50	3.857 [3.508 - 4.239]			1.955 [1.839 - 2.078]				
SR11.1	PCR prev.	High	reference	< 0.0025	PvEBP	reference	< 0.0025			
		Medium	0.859 [0.650 - 1.135]			0.733 [0.524 - 1.027]				
		Low	0.713 [0.555 - 0.917]			0.544 [0.402 - 0.736]				
	Age	2-5	reference			reference				
		6-15	1.349 [1.259 - 1.446]			3.044 [2.676 - 3.463]				
		16-50	2.143 [2.008 -2.288]			10.982 [9.723 - 12.405]				
LSA1.41	PCR prev.	High	reference	< 0.0025	PvDBP	reference	< 0.0025			
		Medium	0.801 [0.606 - 1.058]			0.889 [0.725 - 1.090]				
		Low	0.654 [0.508 - 0.840]			0.768 [0.639 - 0.922]				
	Age	2-5	reference			reference				
		6-15	2.495 [2.208 - 2.818]			1.447 [1.346 - 1.555]				
		16-50	7.547 [6.725 - 8.470]			1.941 [1.813 - 2.078]				
LSA1.J	PCR prev.	High	reference	< 0.0025	Pv.MSP1.19	reference	< 0.0025			
		Medium	0.809 [0.656 -0.998]			0.848 [0.666 - 1.078]				
		Low	0.762 [0.631 - 0.920]			0.751 [0.605 - 0.934]				
	Age	2-5	reference			reference				
		6-15	1.454 [1.351 - 1.564]			1.229 [1.165 - 1.296]				
		16-50	1.875 [1.750 - 2.009]			1.514 [1.440 - 1.593]				
LSA3.RE	PCR prev.	High	reference	< 0.0025						
		Medium	0.678 [0.474 - 0.971]							
		Low	0.472 [0.342 - 0.971]							
	Age	2-5	reference							
		6-15	2.877 [2.528 - 3.273]							
		16-50	16.062 [14.214 - 18.151]							
Pf.MSP1.1	PCR prev.	High	reference	< 0.0025						
		Medium	0.851 [0.664 - 1.089]							
		Low	0.769 [0.615 - 0.961]							
	Age	2-5	reference							
		6-15	1.281 [1.217 - 1.348]							
		16-50	1.651 [1.573 - 1.732]							
GLURP	PCR prev.	High	reference	< 0.0025						
		Medium	0.767 [0.572 - 1.028]							
		Low	0.549 [0.422 - 0.715]							
	Age	2-5	reference							
		6-15	2.396 [2.143 - 2.679]							
		16-50	8.941 [8.046 - 9.935]							
Pf.GLURP.	PCR prev.	High	reference	< 0.0025						
		Medium	0.672 [0.497 - 0.908]							
		Low	0.474 [0.361 - 0.621]							
	Age	2-5	reference							
		6-15	2.702 [2.412 - 3.027]							
		16-50	13.094 [11.765 - 14.577]							

IRR = Incidence Rate Ratio that indicates for how much (if > 1) or less (if < 1) the covariates decline with PCR prevalence level or rise with an increasing age.
This is performed in respect to the reference category and LCI and UCI representing the lower and upper 95% confidence intervals
based on the total sample size of n = 6,499 individuals from 98 villages.
P-value < 0.0025, Bonferroni correction for 20 different tests
Best model for each Ag (model 3): ln(MFI) ~ PCR prev. + age category + (1|Survey/Cluster) (Cluster within Survey as random effect).

GEOGRAPHICAL PATTERNS OF MALARIA TRANSMISSION BASED ON SEROLOGICAL MARKERS FOR FALCIPARUM AND VIVAX MALARIA IN RATANAKIRI, CAMBODIA.

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Abstract

Background: Malaria transmission is highly heterogeneous, especially in low endemic countries, such as Cambodia. This results in small clustered areas of residual transmission in the dry, low transmission season, which can fuel the transmission to wider areas or populations during the wet season. A better understanding of spatial clustering of malaria can lead to a more efficient strategy to reduce malaria transmission. This study aims to evaluate the potential of the use of serological markers to define spatial patterns in malaria exposure.

Methods: Blood samples collected in a community-based randomized trial performed in 98 high endemic communities in Ratanakiri, Cambodia, were screened with a multiplex serological assay for five serological markers (three *Plasmodium falciparum* and two *Plasmodium vivax)*. The antibody half-lives range from approximately six months until more than two years. Geographical heterogeneity in malaria transmission was examined using a spatial scan statistics on serology, PCR prevalence and incidence rate data. Furthermore, to identify behavioural patterns or intrinsic factors associated with malaria exposure (antibody levels), risk factor analyses were performed by using multivariate random effect logistic regression models. The serological outcomes were then compared to PCR prevalence and incidence data.

Results: A total of 6,502 samples from two surveys were screened in an area where the average parasite rate among the selected villages is 3.4%. High-risk malaria pockets were observed adjacent to the 'Tonle San River' and neighbouring Vietnam for all three sets of data (serology, PCR prevalence and incidence rates). The main risk factors for all *P.falciparum* Ags and Pv.MSP1.19 are Age, ethnicity and staying overnight at the plot hut.

Conclusion: It is possible to identify similar malaria pockets of higher malaria transmission together with the possible risk factors by using serology as compared to PCR prevalence and incidence data. People most at risk stayed overnight in plot huts in the field, as well as that the serological markers show that age is an important factor as well. Pf.GLURP.R2 showed a shrinking pocket of malaria transmission over time, and Pf.MSP1.19, CSP, PvAMA1 were also informative for current infection to a lesser extent. Therefore, serology could contribute in future research. However, further in-depth research in selecting the best combination of Ags is required.

1. Background

The annual malaria incidence and mortality have steadily declined in the Greater Mekong Subregion over the past 15 years [1]. In particular, in the Kingdom of Cambodia malaria cases were reduced by more than 75 %, due to the improvements in malaria control by the National Malaria Control Programme, such as free distribution of long-lasting insecticidal nets (LLINs), performing better case management (i.e. diagnosis by RDT and treatment with ACT) [2, 3]. Although malaria transmission in Cambodia nowadays is low [4, 5], still 21 out of 25 provinces are endemic, of which the northeast region (Ratanakiri) accounts for more than 70 % of the malaria burden [3, 6]. Moreover, a shift towards more heterogeneous malaria transmission has been observed. This results in areas that support malaria transmission, which are referred to as foci [7, 8]. Within these foci, elevations in malaria transmission in small areas (sometimes <1 km2 [7]) or populations can be identified, which are respectively called hotspots or hotpops, presenting a higher risk of infection as compared to the rest of the focus [7, 9]. Some studies have shown that stable hotspots with a permanent transmission of the parasite over consecutive dry seasons mainly consist of asymptomatic carriers [10]. Consequently, in low endemic areas as Cambodia, in the wet high transmission season, when the malaria vector population expands, these remaining reservoirs tend to fuel malaria transmission to surrounding areas or populations [7, 11]. Despite considerable malaria control efforts in Cambodia, this persisting transmission makes it impossible to eliminate malaria by current control strategies. Therefore, it is necessary to move forward from these strategies by creating a novel strategic plan based on the understanding of the hotspots/hotpops [3, 12]. It is assumed that there will be no persistence of malaria transmission once these recurrent sources of infection are eliminated by targeted interventions to these hotspots and hotpops [3, 8].

So far, hotspot identification has been carried out by various approaches, including microscopy, rapid diagnostic tests (RDTs), PCR detection and entomological surveys. Microscopy and RDTs cannot detect low-density infections [13]. In addition, among the PCR-based assays, real-time PCR is a highly sensitive technique capable of detecting higher numbers of infected individuals, including asymptomatic infections. However, this technique has a couple of drawbacks when used in the field e.g. high costs and complexity of its applicability [13]. Serological markers of malaria exposure, specifically antibodies (Abs) against *Plasmodium* antigens (Ags), are appropriate to use when detecting stable hotspots of malaria transmission in low endemic areas [7]. These Ab-responses increase by cumulative exposure and the longevity of the Abs depends on the Ag [7]. Therefore, this method can provide an indication of past and

recent malaria exposure that can be used to detect temporal and spatial trends in malaria transmission [7, 14]. Moreover, previous elimination programs have already observed that the absence of Ab-titers in the youngest age-groups could be used as proof of the cessation of malaria transmission [14]. However, in Southeast Asia, primo infection by malaria parasites may be delayed to adolescence, due to behavioural and occupational activities [15]. Serology has already been used to detect spatial trends by previous studies in high endemic settings [8, 11, 16–20]. On the contrary, the serological value for detecting spatial clustering of malaria exposure in low endemic areas has not yet been completely confirmed [7, 8].

The proposed study aims to further validate five *Plasmodium* markers for their potential to detect recent infection [21] by defining spatial patterns in malaria exposure over two different surveys in comparison with PCR prevalence and malaria incidence data. As in this study, community (= cluster) based data were used, the outcomes were defined as 'malaria pockets' [22] referring to an area in between a hotspot (<1 km2 [7]) and a foci (>1 province) where the malaria exposure is higher than the surrounding areas.

2. Methods

2.1 Study area

Ratanakiri province (13°44'N, 107°0'E) bordering Lao PDR and Vietnam, is located 520 km from Phnom Penh in the northeast of Cambodia. The area has a monsoonal climate, resulting in perennial malaria transmission with a peak during the rainy season (April until October) [5, 15]. Ratanakiri has a population of 149,997 individuals spread over 240 villages [23], of which approximately 70 % is living in the highlands and 30 % in the urbanized towns. This study area is largely inhabited by the ethnic minority (e.g. Jarai, Kreung, Tumpuon) as opposed to the Khmer in the rest of the Country. The ethnic minority generate revenue by subsistence slash-and-burn farming. Therefore, they own plot huts located near or inside the forests, where they stay during most of the rainy season [23, 24].

2.2 Sample collection

Samples used were derived from a community (=cluster) based randomized trial (MalaResT project - NCT01663831) that aims to evaluate the effectiveness of topical repellents, in addition to long-lasting insecticidal nets, on malaria prevalence and incidence [5, 21, 25]). For the purpose of this study two cross-sectional surveys carried out in November 2012 and 2013 were included. No differences were observed between the control and the intervention arm for PCR prevalence, serological

indicators and malaria incidence. Blood samples were collected by a finger prick on filter paper, and immediately screened by real-time PCR to determine the presence or absence of parasites [5]. In the MalaResT project 65 people per community were randomly selected, and in case of a low attendance rate an additional set of 15 randomly selected people was added to reach at least 50 participants per community. Throughout the entire sampling process an over 70 % success rate was reached, in which all age groups were proportionally covered [23]. From a total population of 48,838 individuals residing in all 113 villages grouped in 98 communities (i.e. clusters; 88 single villages +25 neighbouring villages having a distance of <1 km were grouped into 10 communities), a total of 6640 and 6715 were randomly recruited by community in November 2012 and 2013 of which respectively 4996 and 5431 were sampled [23]. Serology was performed on 3264 (2012) and 3238 (2013) on randomly selected samples. The PCR *Plasmodium* prevalence was 4.9 and 3.4 % in November 2012 and 2013 respectively (Table 1) [23].

2.3 Serology

Initially, each Ag was coupled to paramagnetic beads (MagPlex microspheres, Luminex Corp, Austin, TX, USA) as described earlier [26]. All beads with different Ags were put together to prepare a microsphere working mixture at a concentration of 1000 beads/Ag/ well. Bloodspot samples were then analysed in 96-well plates in duplicate. Positive controls (pool of four *P. falciparum* and two *P. vivax* infected individuals), negative control serum and blanco (PBS-CR) were added in duplicate to each 96-well plate [21]. The MAGPIX®-system was set for reading a minimum of 400 beads per spectral address and results were expressed as median fluorescent intensity (MFI) [21].

2.4 Antigen selection

The selection of the five Ags used in this study was based on the half-lives estimated on 20 different Ags previously [26]. For *P. falciparum* three out of the six serological markers that were most likely to be reflective for recent exposure were chosen. These were Pf.GLURP.R2, Pf.MSP1.19 and CSP, showing a half-life of respectively six months, ~8 months and ~1 year. For *P. vivax*, the shortest Ab half-life found was more than 1.5 years (PvAMA1 and Pv.MSP1.19).

2.5 Malaria incidence

Passive case detection (confirmed malaria cases) was reinforced for the purpose of the study and relies on the National Health System (Village Malaria Workers, health

centres and hospitals) [6]. For each case the living place was recorded in the database. Incidence was estimated by community.

2.6 Statistical analysis

The serology outcome data (median fluorescent intensity - MFI) were processed and analysed in R-version 3.1.0 [27]. Spatial clustering of serological data was detected for each survey by using spatial scan statistic (SaTScan) [28, 29], which is the preferred software in low risk settings according to Aamodt *et al.* [25, 30]. The spatial scan statistic was used on the natural logarithm transformed median fluorescent intensity values (ln(MFI) - normal probability model) to define spatial patterns in malaria exposure and to compare these outcomes to malaria pockets obtained by the PCR prevalence (binominal, Bernoulli model) and malaria incidence data recorded by community in all ages (counts, Poisson model).

The R package *'rsatscan'* [31] was used to prepare the data, and R package *'PlotKML'* [32] was used to be able to plot the 98 communities on the Google Earth images created with SaTScan. By using the spatial scan statistic, SaTScan version 9.4.2, 64-bit [28, 29], the study area (province of Ratanakiri) was systematically screened for circular windows of higher MFI values. Ratanakiri is a large area (10 782 km²). To avoid detection of too large pockets that cover almost the entire area and hide small homogeneous malaria pockets within the larger pockets, the maximum of the total population at risk within a community was set at 20 percent, in accordance with the observations in the study of Mosha *et al.* [9]. This allows that both small and large malaria pockets can be detected [33]. Observed and expected means of ln(MFI) values inside and outside each window at each location were calculated [8, 11, 29]. The areas with the maximum likelihood were defined as the malaria pockets [11]. These pockets were then examined based on 999 Monte Carlo simulations. The malaria pockets were considered statistically significant with a p-value < 0.05. Circular windows were visualized separately for November 2012 and 2013. The median age inside and outside the malaria pockets was estimated for the serological data.

The outcomes perceived with the serological markers were compared to PCR prevalence (performed on the same surveys) and malaria incidence rate (recorded the same year) [23]. When these outcomes correspond to the results observed in this study, ongoing malaria transmission at the localities could be confirmed. Therefore, these malaria pockets were detected by using a similar approach as detailed above, but with the PCR prevalence data (Bernoulli model) from November 2012 and 2013, whereby the maximum allowed population size was set to 20 percent as well. Thereby,

pockets were significant with a p-value below 0.05. The same was done for the malaria incidence data of symptomatic cases based on the health information system (Poisson model) from 2012 and 2013. Pockets were considered significant when presenting a p-value below 0.05. A sensitivity and specificity analysis was done to assess the prediction of serological based malaria pockets in comparison to PCR or Incidence malaria pockets.

Moreover, a risk factor analysis was performed to detect a pattern in behavioral or intrinsic factors associated with high endemic pockets. The risk factor analysis was carried out in various steps as described previously [25], with minor adjustments. Firstly, a univariate analysis was performed on all explanatory variables: age, gender, ethnicity (khmer *vs* ethnic minority), axillary temperature, plot hut (a human behaviour associated with indigenous farming), sleeping in the forest (indoor/outdoor) and repellent use (control (only bed net) *vs* intervention (bed net and repellent use)) [25]. The risk factors were analysed by fitting linear mixed effect models (lmer function in the *'lme4'* package applied in R version 3.1.0 [34]) with ln(MFI) values as outcome variable taking into account community (= cluster) within survey (year) as random effect [35]. Incidence Rate Ratios (IRR) and 95% confidence intervals (95% CI) were estimated by exponentiation of the model coefficients per variable and per Ag. Statistical significance was evaluated based on the p-values below 0.10. Next, residual plots were made to evaluate how well the models fitted the data and how the data meet the assumptions of the model. To check whether variables needed to be omitted due to co-linearity for the multivariable model, the Variance Inflations Factors (VIF) of each linear model were evaluated (R packages *'MASS'* and *'car'* [36, 37]). Lastly, multivariable random effect logistic regression models were fitted (lmer function in R package *'lme4'* [34]). Model selection through stepwise backward deletion, starting from the full model, was built based on the outcomes of the univariate analysis to define independent relations between the variables and the strength of the Ab-response [38, 39].

3 Results

For this study a random selection of 6,502 samples were screened with the Luminex technology after being tested for parasite infection using real-time PCR. An overview of the descriptive statistics is given in Table 1.

Table 1 Descriptive statistics of the study site and the inhabitants

Characteristics		November 2012	November 2013
No. Samples		3 264	3 238
Age	**2-5**	468 (14.3%)	423 (13.1%)
	6-15	998 (30.6%	968 (29.9%)
	16-50	1793 (54.9%)	1847 (57.0%)
	median	18	19
Gender	**Male**	1610 (49.9%)	1616 (49.9%)
	Female	1653 (50.6%)	1622 (50.1%)
PCR prevalence	**All 98 communities**	4.86%	3.41%
	Pf	2.22%	1.20%
	Pv	2.94%	2.24%
Mean MFI-values Pf markers	**CSP**	208	878
	Pf.GLURP.R2	3064	2075
	Pf.MSP1.19	259	289
Mean MFI-values Pv markers	**Pv.MSP1.19**	277	314
	Pv.AMA1	822	845

3.1 Geographical clustering based on serology (Plasmodium falciparum and Plasmodium vivax antigens)

A first pocket was detected around the most northerly point of the 'Tonle San River' for all five Ags (*P. falciparum* and *P. vivax*) in November 2012 (Figure 1, Table 2). For four out of five Ags (CSP, Pf.MSP1.19, Pf.GLURP.R2 and Pv.MSP1.19), the mean geographical radius was estimated 21.33 km, consisting of approximately 18 communities (p = 0.001). Moreover, for PvAMA1 one pocket was located at the same place, but showed a smaller radius of 16.45 km, with 8 communities (p = 0.001). In the same year, another pocket (Figure 1, Table 2) was perceived around the 'Tonle San River' nearby the border with Vietnam for Pf.MSP1.19, Pf.GLURP.R2 and PvAMA1, with a radius of respectively 5.97 km (5 communities), 2.90 km (2 communities) and 10.67 km (7 communities) (p = 0.001). For the remaining two Ags (CSP and PvMSP1.19), a malaria pocket was detected adjacent to Vietnam as well, but was distant from the 'Tonle San River'. These malaria pockets had a radius of 0 km, as these consist of only one community (p = 0.002). PvAMA1 showed a fourth pocket more inland (3 communities, radius 7 km), and for Pf.MSP1.19 a pocket close to the capital of Ratanakiri, 'Ban Lung', was observed (1 community, radius 0 km).

In November 2014, a malaria pocket was observed again for both Ags Pf.GLURP.R2 and Pv.AMA1, located at the northerly point of the 'Tonle San River' (Figure 1, Table 2). For Pf.GLURP.R2, the pocket size was reduced by approximately 35% (from 18 to 6 communities in the malaria pockets) as compared to November 2012, whereas for PvAMA1 the radius was reduced by 8% (from 8 to 6 communities in the malaria pockets), however, for PvAMA1 the communities neighbouring Vietnam increased with 16.8% (from 7 to 12 communities in the malaria pockets) and showed a more northern spread compared to November 2012. A fourth pocket for Pf.GLURP.R2 was perceived closer to 'Ban Lung' (5 communities, radius 7.54 km).

A different pattern was perceived for CSP, showing an eastward shift (37.35 km) in the malaria transmission locality between November 2012 and 2013 (Figure 1, Table 2). This resulted in only one large malaria pocket (> 1 community) in November 2013, neighbouring Vietnam and having a radius of 12.46 km consisting of 12 communities ($p = 0.001$).

Primary malaria pockets obtained for Pf.MSP1.19 and Pv.MSP1.19 were similar in November 2012 and 2013 (Figure 1, Table 2) having a mean radius of 23.18 km, and 19 communities ($p < 0.001$) with a shift of the main pocket towards the Vietnamese border in November 2013 (mean radius of 27.49 km, 19 communities ($p < 0.001$). Additionally, in November 2013 a smaller malaria pocket appeared for both Ags with a mean radius of 6.44 km, including 6 communities ($p < 0.001$) more to the northwest side of the 'Tonle San River'.

For all Ags the age distribution inside and outside the pockets was shown to be similar (Table 2).

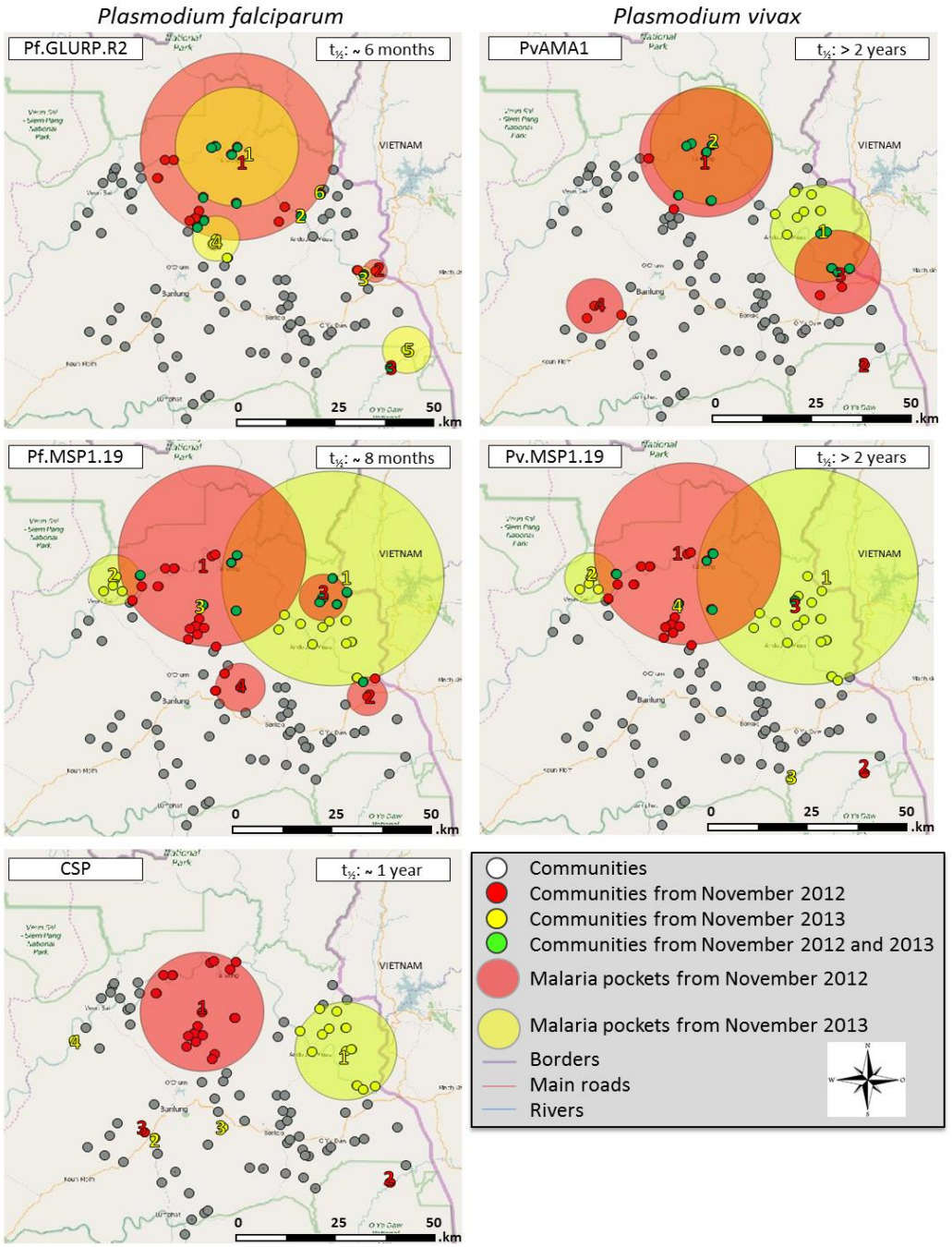

Figure 1: Malaria pockets with higher ln-MFI values for antibodies against *Plasmodium falciparum* and *Plasmodium vivax* in Ratanakiri, Cambodia. White dots indicate all 98 villages that were included in this study. Red dots (November 2012), yellow squares (November 2013) and the green dots (November 2012 and 2013) specify the villages within the most likely clusters. The large red (November 2012) and yellow (November 2013) circles are the statistically significant clusters in which the higher Plasmodium sero-activity was detected by spatial scan (p < 0.005).

Table 2 Spatial malaria pockets with higher level of antibodies against *Plasmodium falciparum* and *Plasmodium vivax* antigens detected by SaTScan v9.4.2 in Ratanakiri, Cambodia.

Antigens	Malaria pockets	Communities (out of 98)	year	Total # persons tested per Ag	# of persons per pocket	% of people living in pocket	Radius (km)	Mean ln-MFI inside pocket	Mean ln-MFI outside pocket	Median age inside pocket	Median age outside pocket	Log likely-hood ratio	p-value
CSP	3	17	Nov 2012	3067	540	17.6%	15.15	5.69	5.37	20	18	12.88	0.001
		1			18	0.6%	0*	6.84	5.42	16	18	10.54	0.002
		1			23	0.7%	0*	6.53	5.43	20	18	8.15	0.018
	4	12	Nov 2013	2880	335	11.6%	12.46	5.77	5.41	16.5	18	11.67	0.001
		1			11	0.4%	0*	6.95	5.45	25	18	7.81	0.018
		1			20	0.7%	0*	6.54	5.45	16	18	7.48	0.026
		1			5	0.2%	0*	7.59	5.45	20	20	7.20	0.034
Pf.MSP1.19	4	19	Nov 2012	2921	563	19.3%	23.18	5.46	5.21	20	18	91.87	0.001
		3			88	3.0%	4.95	5.64	5.28	22	18	11.59	0.001
		5			163	5.6%	5.97	5.53	5.28	15	18	10.05	0.001
		3			84	2.9%	6.16	5.58	5.29	18	18	7.68	0.018
	4	19	Nov 2013	2757	549	19.9%	27.49	5.68	5.31	16	19	60.45	0.001
		6			162	5.9%	6.44	5.69	5.37	18	18	16.10	0.001
		1			26	0.9%	0*	6.05	5.38	26	18	11.23	0.001
		1			32	1.2%	0*	5.88	5.38	23	18	7.83	0.017
Pf.GLURP.R	4	18	Nov 2012	3142	601	19.1%	23.79	7.18	6.60	19	18	26.52	0.001
		2			59	1.9%	2.90	8.01	6.69	20	18	16.77	0.001
		1			26	0.8%	0*	8.35	6.70	16	18	11.49	0.002
		1			37	1.2%	0*	7.89	6.70	14	18	8.50	0.009
	6	6	Nov 2013	2953	177	6.0%	15.15	7.32	6.54	19	18	19.29	0.001
		1			40	1.4%	0*	7.87	6.57	19	18	12.90	0.002
		2			44	1.5%	1.92	7.60	6.57	20	18	8.87	0.010
		5			166	5.6%	5.74	7.07	6.56	19	18	7.93	0.020
		2			68	2.3%	5.97	7.33	6.57	18	18	7.33	0.032
		1			20	0.7%	0*	7.97	6.57	13	18	7.33	0.032
PvAMA1	4	8	Nov 2012	3030	264	8.7%	16.45	6.64	6.20	20	18	32.10	0.001
		1			21	0.7%	0*	7.61	6.23	16	18	27.25	0.001
		7			200	6.6%	10.67	6.52	6.21	18.5	18	11.74	0.001
		3			56	1.8%	7.00	6.70	6.23	20	18	8.35	0.025
	2	12	Nov 2013	2801	324	11.6%	12.46	6.66	6.25	16.5	18	36.73	0.001
		6			182	6.5%	15.15	6.64	6.28	19	18	16.39	0.001
Pv.MSP1.19	3	19	Nov 2012	2915	564	19.3%	23.18	5.64	5.24	20	18	74.18	0.001
		1			12	0.4%	0*	6.41	5.31	16	18	14.45	0.001
		1			30	1.0%	0*	5.99	5.31	13	18	13.95	0.001
	4	19	Nov 2013	2762	547	19.8%	27.49	5.69	5.35	16	19	48.55	0.001
		6			161	5.8%	6.44	5.71	5.40	18	18	13.32	0.001
		1			34	1.2%	0*	5.94	5.41	23	18	8.54	0.012
		1			27	0.97%	0*	5.96	5.41	26	18	7.30	0.029

3.2 Confirmation of the ongoing malaria transmission

Plasmodium prevalence and malaria incidence were determined by PCR (prevalence per survey, Figure 2, table 3) [25] and passive case detection (yearly incidence rate, Figure 2, Table 4). The same pockets as the ones detected by serological markers were observed with PCR in the most northerly area of the 'Tonle San River' in November 2012 for *P. falciparum*, whereas no PCR prevalence pockets were detected in November 2013. Looking at the *P. falciparum* malaria incidence data, malaria pockets did not move between 2012 and 2013, but slightly shifted to the western part of the river as compared to the main pocket acquired with PCR prevalence and serological markers.

For *P. vivax* sero-reactive pockets were located nearby the 'Tonle San River' and the border of Vietnam in November 2012. Malaria pockets detected by PCR prevalence data show a shift between the surveys from the river-side towards the south (inland) of Ratanakiri in November 2013, the same pocket was observed for PvAMA1 in November 2012. This shift to the south is also seen with the malaria incidence data but on a different locality namely at the border of Vietnam. However, in 2013 (malaria incidence data) the main pocket persisted at the northern site of the 'Tonle San River', which was not the case for PCR prevalence data.

Overall, malaria pockets found by analysing malaria incidence data and ln(MFI) data are similar. The largest serologically measured pocket of each Ag consistently overlaps with the largest incidence based pockets. The malaria pockets adjacent to Vietnam overlap for all three approaches. Moreover, serologically obtained pockets from November 2012 show overlap with both *P. falciparum* and *P. vivax* pockets found by PCR. In November 2013, no significant PCR prevalence based pockets were observed for *P. falciparum* and the largest PCR prevalence based pocket for *P. vivax* was located more inland.

3.3 Sensitivity and specificity (Additional file 1: Table S1)

Serology with the three *P. falciparum* Ags provides a relative good specificity ($\geq$74%) and sensibility ($\geq$72%) to identify positive PCR falciparum pockets. This was particularly true in November 2012 with the Pf.GLURP.R2 Ag having 95% for both specificity and sensitivity. As no PCR falciparum pockets could be identified for November 2014, sensitivity could not be calculated. To predict *P. falciparum* incidence pockets, serological data provide a much lower sensitivity (between 13 and 60%) although specificity was around 80%. Serological data with the two *P. vivax* Ags provides a low sensitivity (between 0 and 38%) but a relatively high specificity (69% and 82%) to

predict PCR vivax pockets. Results were slightly better in predicting *P. vivax* incidence pockets (sensitivity 10% and 57%; specificity around 80%). For comparison *P. falciparum* and *P. vivax* incidence data to predict PCR falciparum and vivax pockets sensitivity was around 50% in 2012, but only 10% in 2013 for *P. vivax*. Specificity lies between 70% and 80%.

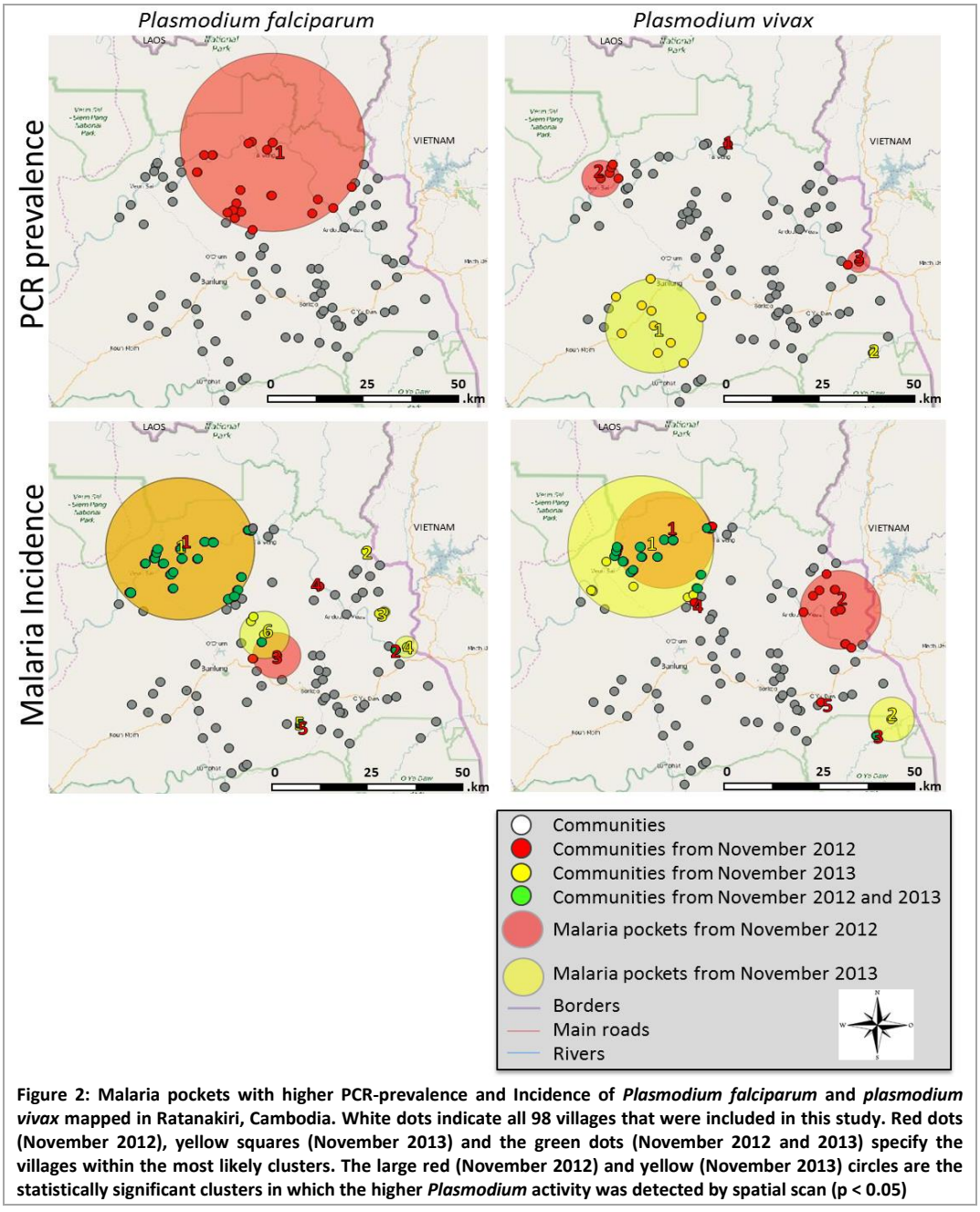

Figure 2: Malaria pockets with higher PCR-prevalence and Incidence of *Plasmodium falciparum* and *plasmodium vivax* mapped in Ratanakiri, Cambodia. White dots indicate all 98 villages that were included in this study. Red dots (November 2012), yellow squares (November 2013) and the green dots (November 2012 and 2013) specify the villages within the most likely clusters. The large red (November 2012) and yellow (November 2013) circles are the statistically significant clusters in which the higher *Plasmodium* activity was detected by spatial scan (p < 0.05)

Table 3 Spatial malaria pockets with higher PCR-prevalence of *Plasmodium falciparum* and *Plasmodium vivax* species detected with SaTScan v9.4.2 in Ratanakiri, Cambodia.

Plasmodium Species	Malaria pockets	Communities (out of 98)	year	Radius (km)	Population	Observed cases	Expected cases	Relative risk	Log likelihood ratio	p-value
P. falciparum	2	19	Nov 2012	23.92	953	46	21.36	2.96	14.75	< 0.05
		1		0*	53	7	1.19	6.22	7.10	< 0.05
	0		Nov 2013							> 0.05
P. vivax	3	1	Nov 2012	0*	52	12	1.55	8.33	15.66	< 0.05
		5		4.91	259	22	7.72	3.17	9.93	< 0.05
		2		2.90	103	13	3.07	12.6	9.69	< 0.05
	2	1	Nov 2013	0*	61	10	1.38	7.79	12.15	< 0.05
		7		12.69	538	29	12.18	2.81	9.98	< 0.05

*A single village was selected as an area with a higher risk to *Plasmodium* infection, and therefore showing a radius of 0 km

Table 4 Spatial malaria pockets with higher Incidence of Vivax and Falciparum malaria detected by SaTScan v9.4.2 in Ratanakiri, Cambodia.

Plasmodium Species	Malaria pockets	Communities (out of 98)	year	Radius (km)	Population	Observed cases	Expected cases	Relative risk	Log likelihood ratio	p-value
P. falciparum	5	27	2012	19.08	9594	1113	527.11	2.92	335.44	< 0.05
		1		0*	244	57	13.41	4.32	39.27	< 0.05
		3		6.16	699	102	38.40	2.72	36.82	< 0.05
		1		0*	288	44	15.82	2.81	16.97	< 0.05
		1		0*	223	33	12.25	2.71	12.03	< 0.05
	6	27	2013	19.08	9594	580	257.69	3.26	204.42	< 0.05
		1		0*	450	47	12.09	4.00	29.39	< 0.05
		2		1.50	535	41	14.37	2.91	16.63	< 0.05
		2		2.90	876	55	23.53	2.40	15.62	< 0.05
		1		0*	223	22	5.99	3.72	12.71	< 0.05
		5		6.36	325	21	8.73	2.43	6.22	< 0.05
P. vivax	5	19	2012	13.01	6752	464	201.37	2.92	154.77	< 0.05
		9		10.59	3408	173	101.64	1.80	22.59	< 0.05
		1		0*	283	30	8.44	3.61	16.64	< 0.05
		1		0*	222	24	6.62	3.67	13.63	< 0.05
		1		0*	368	26	10.97	2.39	7.48	< 0.05
	2	27	2013	19.08	9594	366	154.30	3.60	145.45	< 0.05
		2		5.97	386	31	6.21	5.16	25.46	< 0.05

*A single village was selected as an area with a higher risk to *Plasmodium* infection, and therefore showing a radius of 0 km

3.4 Risk factor analysis

Significant variables (age, gender, ethnicity, plot hut and sleeping in the forest) observed in the univariate analysis (Additional file 2: Table S2) were further explored in the multivariable regression model (Table 5). In the full model used for the multivariable analysis age, gender, ethnicity, plot hut and sleeping in the forest were included as dependent variables, and community within survey as random effect. Axillary temperature and repellent were not significant for all five Ags in the univariate exploration and were, therefore, not included.

Differences in the presence of Abs were perceived between age categories for all Ags. Ethnic minority showed a higher presence of Ab-levels for all Ags (mean IRR 2.051 95%CI[1.788 – 2.354]). Significant differences were observed for 'overnight stay in a plot hut' ($p < 0.10$) with a mean IRR and 95%CI of 1.126 [1.067-1.188]. Furthermore, gender appeared to be significantly different for CSP, Pf.MSP1.19 and Pv.MSP1.19, and sleeping in the forest for CSP and Pv.MSP1.19. However, the IRR of these last two risk factors are so close to one, that this is probably negligible.

Table 5 Multivariable analysis of the selected risk factors associated with the seroprevalence after the univariate analysis and stepwise backward deletion.

	Plasmodium falciparum			*Plasmodium vivax*	
Malariometric variable	**CSP**	**Pf.MSP1.19**	**Pf.GLURP.R2**	**PvAMA1**	**Pv.MSP1.19**
Variable level	**IRR* [LCI-UCI]**	**IRR* [LCI-UCI]**	**IRR* [LCI-UCI]**	**IRR* [LCI-UCI]**	**IRR* [LCI-UCI]**
Age (years)					
2-5	reference	reference	reference	reference	reference
6-15	1.44 [1.33 - 1.57]	1.30 [1.23 - 1.38]	2.83 [2.54 - 3.15]	1.40 [1.31 - 1.50]	1.21 [1.14 - 1.28]
16-50	5.44 [5.03 - 5.89]	1.68 [1.59 - 1.78]	13.33 [12.02 - 14.77]	1.98 [1.86 - 2.16]	1.50 [1.42 - 1.59]
> 50	11.82 [10.61 - 13.15]	2.01 [1.86 - 2.16]	17.90 [15.55 - 20.61]	2.79 [2.55 - 3.05]	1.99 [1.84 - 2.15]
Gender					
Male	reference	reference	.	.	reference
Female	1.06 [1.01 - 1.12]	1.04 [1.00 - 1.08]	.	.	1.06 [1.02 - 1.10]
Ethnicity					
Khmer	reference	reference	reference	reference	reference
Ethnic Minority(EM)	2.72 [2.38 - 3.12]	1.33 [1.21 - 1.47]	3.70 [3.11 - 4.41]	1.31 [1.17 - 1.47]	1.18 [1.07 - 1.31]
Overnight plothut					
No	reference	reference	reference	.	reference
Yes	1.12 [1.06 - 1.19]	1.07 [1.03 - 1.11]	1.24 [1.15 - 1.33]	.	1.04 [1.00 - 1.09]
Overnight forest					
No	reference	.	.	.	reference
Yes	1.14 [1.05 - 1.23]	.	.	.	1.07 [1.01 - 1.14]

*IRR = Incidence Rate Ratio that indicates for how much (if > 1) or less (if < 1) the risk factors affect the data obtained in survey 2 (2012) and survey 4 (2013). This is performed in respect to the reference category and LCI and UCI representing the lower and upper 95% confidence intervals based on the total sample size of n = 6 502 individuals from 98 communities. p-value < 0.10. Missing values were not significant.

4 Discussion

Methods that can identify stable areas of transmission over time are suggested to be most effective for assessing geographical variations in malaria exposure. Therefore, Ab-responses acquired with cumulative malaria exposure, measured over several seasons, were recommended for implementation in geographical clustering analyses [7]. During the '90s geographical cluster analyses mainly relied on symptomatic cases with accurate details about the place of infection and/or residence [20, 40], and were merely based on passive case detection (PCD) [25]. It was not until the 21th century that due to a lack of information about the parasite reservoir in asymptomatic cases [10], new studies arose focussing on the spatial distribution estimated with PCR-

prevalence data of species-specific geographical areas of infections based on asymptomatic carriers [26]. This approach was especially important in countries with a low endemicity where the majority of infected people are asymptomatic carriers [25]. Another innovative approach is the application of serological markers in defining these geographical areas. Serology already proved its ability to improve predictions of low transmission risk [26, 41].

Where most studies only focused on PfAMA1 and PfMSP1.19 [8, 11, 16–19] the advantage of this study is the amount of additional Ags from both falciparum and vivax malaria investigated, compared to most other studies. Only one previous geostatistical study has used several Ab markers (namely PvAMA1, PvMSP1.19, PfAMA1 and Pf.GLURP.R2). However, in contrast with the current study, the researchers considered an individual positive when it responded for any of the two Ags for each species, not taking into account the differences in biological activity (e.g. longevity) among these Ags [20].

The previous study performed by Kerkhof *et al.* [26] has led to identification of serological markers with a relatively short half-life that were most likely to be reflective for recent exposure, such as *P. falciparum* Ags Pf.GLURP.R2, Pf.MSP1.19 and CSP. These serological markers could map the transmission risks with more precision and accuracy, as they provide the ability of distinguishing recent from past exposure [26, 41]. The current study, presented here, explored whether or not the use of serological markers is comparable to the use of PCR prevalence (asymptomatic cases) and malaria incidence data (symptomatic cases) to investigate spatial patterns in malaria transmission.

In Ratanakiri, significant malaria pockets were observed for both *P. falciparum* and *P. vivax* Ags. The largest pockets were located around the most northerly site of the 'Tonle San River' for all Ags. In comparison with the PCR prevalence data, *P. falciparum* exhibited similar pockets, whereas for *P. vivax* differences were seen. The similar pockets found between the PCR prevalence rates and sero-reactivity are in line with a study performed by Bousema *et al.* [16], that observed tight correlations as well.

When comparing the serologically based pockets with the incidence based pockets, the pockets neighbouring Vietnam were comparable, while the most northerly pockets at the 'Tonle San River' were slightly shifted to the West. There are malaria incidence based pockets found for *P. falciparum* situated around the capital 'Ban Lung' of the Ratanakiri province. Different studies [24, 42, 43] investigated the movement of

individuals between villages, districts and countries. This might explain the malaria pockets seen around 'Ban Lung' raising the possibility that these individuals travel occasionally towards communities nearby the river or to remote areas. Overall, overlap was seen in the serological based pockets compared to the malaria incidence and parasite prevalence data.

That most pockets were perceived around the river confirms findings from other studies that also found more malaria pockets along open water bodies [19, 20, 44, 45]. The same pattern was observed by Sluydts *et al.* [25] who suggests that this is perhaps associated with increased movements of infected individuals and mosquito populations along the 'Tonle San River', and with the more remote location of these villages [25].

The specificity of serological markers for *P. falciparum* and *P. vivax* was acceptable (between 72% and 95%) to predict PCR and malaria incidence pockets. However, sensitivity was in general much lower, except in predicting *P. falciparum* PCR pockets (between 74% and 95%). In comparison, sensitivity of *P. falciparum* and *P. vivax* incidence in predicting PCR pockets lies between 10% and 50%.

When looking at the different serological markers, variable patterns were observed, going from malaria pockets that move between the east and west in November 2012 and 2013 (CSP, Pf.MSP1.19 and Pv.MSP1.19) to lasting pockets that became smaller (Pf.GLURP.R2) or remained similar in size (PvAMA1). These varying patterns require further investigation related to the differences in immunogenicity and persistence of the Ab-responses [11, 46]. The only Ag that follows an expected altering pattern over time was Pf.GLURP.R2, which seems to correlate best with the PCR-prevalence and malaria incidence data. The latter is probably explained by the fact that this is a blood stage Ag with a short estimated Ab half-life [26]. This might reflect recent exposure with observing pockets that decline over time, suggesting that this serological marker might have potential in evaluating targeted malaria control efforts.

Risk factors related to sero-reactivity were identified by univariate and multivariable analyses. Significant elevated risks for *P. falciparum* malaria were seen for age, ethnicity and overnight stay at the plot hut. There were also differences observed in gender and sleeping in the forest, however, this is most probably negligible, as the IRR was very close to 1. Significant elevated risks for vivax markers were seen for age, whereas staying in plot huts showed to be a risk factor for Pv.MSP1.19 only. The *P. falciparum* outcomes are in line with a previous study performed in the same area by

Sluydts *et al.* [25]. In this PCR prevalence based study that was performed on the baseline survey during the dry season, the most important risk factor detected was the overnight stay in the plot hut, based on both univariate and multivariable analyses. However, in the current serologically based study, it seems that age, concerning the older age groups, was the most important factor determining Ab-levels, compatible with cumulative exposure [7, 17, 26]. When immunity is acquired these Abs can persist for several years. This is caused by the presence of long-lived plasma cells that with every new exposure rapidly produce Abs against these parasites [47]. Therefore, when defining current exposure it is important to observe the Ab-levels in especially the younger age groups [17, 47]. Differences between *P. falciparum* and *P. vivax* could be explained by that fact that *P. vivax* shows relapsed patterns that influence the serological outcomes, and that longer half-lives were observed for the *P. vivax* Ags in a previous study [20].

Overall, these outcomes confirm the findings of Sluydts *et al.* [25], and are also in line with the findings of Incardona *et al.* [48]. These researchers mentioned that entire families go together to the field and sleep in plot huts resulting in an increased exposure risk [25, 48]. Although this is not related to the age differences, as the age composition was similar inside and outside the pockets. However, this can be explained by the immunological maturity-status where children that acquire a malaria infection have the ability to boost their IgG titers, followed by a rapid decay [49]. These outcomes explain the population characteristics in the Greater Mekong Subregion, where ethnic minority groups, forest workers (of all ages) and migrants are known as the most widely recognized groups at risk [25, 50].

This study contributes in the validation of serological markers to distinguish very recent from past exposure, as suggested by Sturrock *et al.* [41]. This is especially the case for Pf.GLURP.R2, but also for Pf.MSP1.19, CSP and PvAMA1. By this means, combining more different Ags, covering the entire *Plasmodium* life cycle and having a longevity ranging from very short (~1 months) to long (year round), might lead to other promising results. However, methods to acquire the exact Ab-persistence are still in its infancy [201]. While PvAMA1 showed stable malaria pockets, Pf.GLURP.R2 suggest a decline in the remaining malaria pockets. The stability of pockets was also observed by Mosha *et al.* [11] on *P. falciparum* Ag AMA1 in the high endemic setting of Tanzania. Further development in quantifying exposure over different timescales, as well as the measurement of very recent exposure, serological approaches will provide a major contribution in estimating spatio-temporal patterns of risk [41]. The use of serology could benefit future malaria control programmes, since the use of serological

markers can more precisely identify variation in transmission in low endemic areas. It should be noted that more serological markers that are competent to estimate exposure over different time-scales are required, as at present Pf.GLURP.R2 is most informative [41], as well as Pf.MSP1.19, CSP and PvAMA1 to a lesser extent. However, Pv.MSP1.19 should certainly not be ruled out, as it probably reflects transmission in the former past for which no PCR-prevalence data may be available.

5 Conclusion

Identification of pockets with higher malaria transmission would be essential when adopting a malaria elimination strategy. Present study shows that PCR parasite prevalence, malaria incidence rates or serology, show equivalent results in identifying malaria pockets. The attempt to validate serological markers that are most likely to reflect current and past exposure led to Pf.GLURP.R2 showing a shrinking malaria pocket over time. Moreover, Pf.MSP1.19, CSP and PvAMA1 are also reflective for recent malaria transmission to a lesser extent. This means that serology can provide promising information for future research, especially in evaluating short-term interventions for malaria elimination. However, there is still more research required in selecting a promising combination of Ags, in particular for *P. vivax*.

Declarations

Ethics approval and consent to participate

The study protocol was reviewed and approved by the Cambodian National Ethics Committee on Health Research (Approval 265 NECHR), the Institutional Review Board of the Institute of Tropical Medicine Antwerp (Approval IRB/AB/ac/154) and the Ethics Committee of the University of Antwerp (Approval B300201112714). Gatekeepers provided informed written consent for the participation of their village. The survey participant or his/her parents or guardian provided informed written consent for individual participation.

Consent for publication

Not applicable

Availability of data and material

The datasets supporting the conclusions of this paper are included within the paper. Raw data may be obtained from the corresponding author on request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Sample collection of the survey was performed by the CNM team (National Centre for Parasitology, Entomology and Malaria Control), the ITM Antwerp team and the Institut Pasteur du Cambodge (IPC) team in Ratanakiri Province in Cambodia. Screening of the blood spot samples was performed at the IPC by KK and LW and SB. The study design and literature research has been performed at the ITM by KK, MP, LD, VS and MC. Data entry and quality control was performed at the ITM by KK, LD and VS. Statistical analyses were performed by KK, LD and VS. The first draft of the manuscript was written by KK. All authors read and approved the final manuscript.

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7 Additional files

Additional file 1 Sensitivity and specificity analysis for the identification of malaria pockets.

P. falciparum		CSP		Pf.MSP1.19		Pf.GLURP.R2	
Reference	Survey	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
PCR	S2	0.74	0.95	0.84	0.82	0.95	0.95
	S4	NA	0.85	NA	0.72	NA	0.83
Incidence	S2	0.28	0.85	0.6	0.79	0.4	0.84
	S4	0.13	0.84	0.37	0.76	0.2	0.84

P. vivax		Pv.MSP1.19		PvAMA1	
Reference	Survey	Sensitivity	Specificity	Sensitivity	Specificity
PCR	S2	0.13	0.78	0.38	0.82
	S4	0	0.69	0	0.79
Incidence	S2	0.39	0.84	0.3	0.84
	S4	0.57	0.81	0.1	0.79

Reference	Survey	Incidence	
		Sensitivity	Specificity
PCR (Pf)	S2	0.47	0.8
	S4	NA	0.69
PCR (Pv)	S2	0.5	0.79
	S4	0.09	0.77

Additional file 2 Univariate analysis of risk factors per antigen and per *Plasmodium* species in Ratanakiri, Cambodia (2012 and 2013)

	Plasmodium falciparum			*Plasmodium vivax*	
Malariometric variable	**CSP**	**Pf.MSP1.19**	**Pf.GLURP.R2**	**PvAMA1**	**Pv.MSP1.19**
Variable level	**OR[LCI-UCI] p-value**	**OR[LCI-UCI] p-value**	**OR[LCI-UCI] p-value**	**OR[LCI-UCI] p-value**	**OR[LCI-UCI] p-value**
Age (years) NA=5	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
2-5	reference	reference	reference	reference	reference
6-15	1.508 [1.391 - 1.635]	1.290 [1.226 - 1.358	2.824 [2.538 - 3.143]	1.406 [1.319 - 1.500]	1.218 [1.153 - 1.287]
16-50	5.867 [5.436 - 6.331]	1.693 [1.612 - 1.777]	13.758 [12.440 - 15.215]	1.983 [1.867 - 2.107]	1.545 [1.466 - 1.627]
> 50	12.719 [11.448 - 14.132]	1.977 [1.847 - 2.117]	18.529 [16.118 - 21.301]	2.760 [2.537 - 3.002]	2.040 [1.898 - 2.194]
Gender NA=1	**< 0.001**	**< 0.001**	0.126	**0.018**	**0.006**
Male	reference	reference	reference	reference	reference
Female	1.140 [1.069 - 1.215]	1.066 [1.031 - 1.102]	1.066 [0.982 - 1.157]	1.051 [1.008 - 1.096]	1.050 [1.014 - 1.088]
Ethnicity NA=22	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**0.002**
Khmer	reference	reference	reference	reference	reference
Ethnic Minority	2.507 [2.111 - 2.976]	1.302 [1.190 - 1.425]	3.115 [2.505 - 3.873]	1.248 [1.115 - 1.397]	1.165 [1.059 - 1.283]
Axillary Temp NA=8	0.026	0.903	0.016	0.529	0.898
< 37.5	reference	reference	reference	reference	reference
≥ 37.5	1.215 [1.023 - 1.442]	0.994 [0.909 - 1.088]	1.311 [1.053 - 1.632]	0.965 [0.862 - 1.079]	1.006 [0.915 - 1.107]
Overnight plothut NA=25	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
No	reference	reference	reference	reference	reference
Yes	1.472 [1.373 - 1.578]	1.129 [1.088 - 1.172]	1.780 [1.628 - 1.945]	1.175 [1.122 - 1.229]	1.108 [1.066 - 1.152]
Overnight forest NA=28	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
No	reference	reference	reference	reference	reference
Yes	1.937 [1.766 - 2.124]	1.169 [1.113 - 1.228]	2.609 [2.319 - 2.935]	1.233 [1.159 - 1.310]	1.177 [1.118 - 1.240]
Arm NA=0	0.377	0.302	0.447	0.574	0.817
Control	reference	reference	reference	reference	reference
Intervention	1.061 [0.931 - 1.209]	0.936 [0.826 - 1.061]	1.085 [0.879 - 1.340]	1.033 [0.921 - 1.159]	0.986 [0.877 - 1.109]

GENERAL DISCUSSION

1. Background and aims

Malaria is one of the foremost causes of death from infection diseases. Despite large progress in malaria control, malaria remains an important public health concern. For countries heading to elimination, such as Cambodia, evaluation and implementation of additional malaria control tools is crucial. However, follow-up of malaria prevalence and incidence using classical malariometric tools becomes challenging and limit the applicability in low malaria transmission settings. To that end researchers started to focus on epidemiological studies based on serology, as serology can be used to assess malaria endemicity and observe current, recent and past malaria exposure when parasite rates are low or even zero [1]. Not until the 1960s, when the ImmunoFluorescent Antibody test (IFAT) [2–5] came in sight, the interest in evaluating malaria transmission by means of the detection of antibodies (Abs) against malaria antigens (Ags) arose. This tool provided good results, however alas, almost only for *P. falciparum.* This is related to the fact that IFAT takes into account the whole antigenic battery [6]. Therefore, Ags are obtained after in vitro culturing of the *Plasmodium* parasite, which was so far only successful for *P. falciparum* [7, 8]. This tool became even more cumbersome due to the lack of reproducibility, the possibility to automate this tool, the skilled personal needed for the fluorescent microscope and the large volumes of sample volume required [9, 10]. The demand for a tool containing these features grew and led ultimately to the ELISA [5, 9, 11], making it possible to include different *Plasmodium* species [8]. Eventually, researchers tried to screen more and more Ags, resulting in a massive amount of serum sample volumes needed, as the amount of 96-well plates per additional Ag increased. However, with more countries moving towards a pre-elimination phase, it became logistically and ethically more challenging to collect large numbers of blood sample [12]. Hence the interest for a multiplex serological assay (xMAP® system (Luminex)) [13] capable of screening more than one Ag simultaneously in one batch of serum sample volume arose.

The arrival of the Luminex is one of the reasons that serology was applied as a secondary indicator on samples collected within the large-scale project (MalaResT) in which this thesis is embedded. This has contributed to the formulation of the following objectives of this thesis:

- To review the malaria transmission intensity and epidemiology based on serological markers with the classical serological tools (IFAT) versus the modern tool (Luminex) (**Chapter 3**)
- To implement a highly performant, standardized and reproducible serological test for detecting multiple serological markers for malaria infection by different parasites (**Chapter 4**)

- The use of the multiplex immunoassay to improve the understanding of the effectiveness of the additional use of topical repellent on malaria incidence in a community based trial (**Chapter 5**)
- To document the half-life and to define the sensitivity of serological markers to pick up recent changes in malaria transmission over time by analyzing sequential and cohort samples from individuals in Ratanakiri, Cambodia (**Chapter 6**)
- To evaluate the potential of the multiplex serological assay in identifying the remaining malaria pockets of malaria transmission (**Chapter 7**)

2. Technical suitability of the proposed multiplex immunoassay

To our knowledge this is the first multiplex immunoassay performed on malaria in the Greater Mekong Subregion (GMS), and the first study which includes 20 different antigens (Ags) directed against three *Plasmodium* species (*P. falciparum*, *P. vivax,* and *P. malariae*) and specific for *Anopheles gambiae* salivary glands. The latter are used as no other salivary gland serological markers are available. Even though, it was still interesting to evaluate the immune responses against the *An. gambiae* Ags. Several studies implemented the multiplex immunoassay for serology and perceived similar outcomes as compared to ELISA [14–17]. From this prominent tool many practical advantages emanated. The possibility of screening multiple Abs simultaneously limited the amount of 96-well plates and serum sample volume, causing a reduction in time, measurement and costs. This was confirmed by several studies [14–16, 18–20], as well as in **Chapter 4**. In addition, the reproducibility of this assay was a major improvement compared to IFAT that is not good reproducible. Besides all these advantages, there is a limitation, as there is no true 'gold standard' for the multiplex immunoassay, and most studies rely on comparing their outcomes with PCR-prevalence or ELISA. This requires more in-depth investigation. Despite this the implemented immunoassay in **Chapter 4** proved to have a very good intraplate, interplate and intercoupling reproducibility, as confirmed by previous studies [14, 15, 21–23] as well. Another key aspect is the flexibility of including new serological markers, without the need to exclude the preceding markers [15]. In addition, Sarr *et al.* [19] observed that the assessment of the force of infection became more precise with the use of multiple Ags, as cumulative exposure may be better displayed, though these statistical approaches on serological data are still in its infancy.

3. Cross-sectional and longitudinal results of the multiplex immunoassay as compared to PCR and clinical incidence

Up to this moment multiple studies combined short- and long-lived serological markers for *Plasmodium* to increase the knowledge about past and recent changes in

malaria transmission between different areas [24–26]. The latter has also been seen by other studies that found a pattern in which Ab-levels drop or even disappear when re-infections are absent [27–29], and in which these levels increase parallel with age and re-infections [30–32]. A variety of mathematical models for serology have been developed to obtain a force of infection of malaria transmission in a given area, and from this, researchers subsequently began to focus on estimating the half-life of Ab-responses. The latter was also estimated in **Chapter 6** for all 20 different serological markers used in this thesis.

The most widely used models in the context of seroepidemiology were built upon a model developed by Muench *et al.* [33]. Thereafter, Draper *et al.* [34] was the first to introduce this model on malaria. This model, a reversible catalytic model, assumes one single seroconversion and seroreversion rate for all age group within a population during the wet transmission season. Additionally, the model suggests that naïve and seropositive people return to being seronegative with a constant force of infection rate [26]. Through the years that followed there were a few (if any) studies that confirmed or refuted these outcomes. Finally, several studies [26, 35–37] applied this model after some adjustments, in which they used the data from endemic settings (cross sectional data) and a threshold model (positive *vs* negative people) to define a cut-off per serological marker. When the cut-off is defined, the model will provide a proxy for malaria transmission as an output, in which the seroconversion rate is closely related to the force of malaria infection [36]. In **Chapter 4** was seen that the estimated seroreversion rates obtained with this model are likely not very reliable, as large 95% confidence intervals were observed. However, it was possible to perceive differences in seroconversion rates over time between the different serological markers used. Although, these models allow a rapid and local evaluation of malaria transmission intensity as seen in Chapter 4 and in other studies [26, 35, 38], the use of a threshold between exposed and non-exposed individuals lacks standardization as these binary outcomes might bias the estimates [39].

Growing forth from this knowledge, many other models were developed using the catalytic model on the basis. An important first step forward was the inclusion of time, age that allow to observe changes in malaria exposure [40]. This was a model that combined the obtained malaria transmission rates from cross-sectional surveys, with an Ab-decay to serological data collected in longitudinal surveys [39]. However, they related the Ab-decay solely with the Ab persistence at high levels in an individual and not with age. This model was followed by means of a stochastic model [41] taking into consideration age, Ab-acquisition and the exponential Ab-decay. The model is applied

on cross-sectional data, in which the parasite and seroprevelance are taking into account.

Through the evolution of serological modelling the majority of the models are based on a fixed threshold value (positive *vs* negative). To this extent, these models needed to be elaborated taking the biological activity of Abs as well as the possibility that an individual stays seropositive for a long time into consideration [42]. A finite mixture model that decomposes a range of Ab-titres was developed by Bretscher *et al.* [39]. The latter is capable of estimating the seroconversion rate and transmission intensity without the use of fixed values. With this model they move a step forward by proving a method without a threshold is more robust and provides more information, but on the other hand, it can only be applied on longitudinal data. Therefore, in the current settings, there is need to develop a serological statistical method that is capable of taking into account the biological activity of an Ab, age and time, as well as is capable of using both longitudinal and cross sectional data. Alternatively, researchers started to use linear regression models [24, 43], generalized estimated equation models [18, 20], or Ab-intensity models [44] to obtain information about the Ab acquisition in populations (cross sectional data) and at individual level (follow-up studies) [24, 45]. In contrast to the models as described before they limited the biased estimates by creating a more sensitive detection of changes in malaria transmission and enhance the precision in estimating malaria transmission intensity [44]. To this end, methods outlined in **Chapter 6** that also do not require a cut-off value to differentiate between seropositive and seronegative individuals have proved advancement in the evolution of seroepidemiological measurements. In **Chapter 6** three different approaches were used that focused on linear regression and generalized estimate equation models to estimate Ab half-lives for all 20 serological markers as well. The use of more than one approach improves the robustness of the results. The first approach in **Chapter 6** made this study even stronger, because if a serological marker could observe a difference between the PCR positive *vs.* the PCR negatives, this marker is capable of picking up a malaria infection (recent or not recent). With this approach the Ags that are not reflective for current infection can be discarded, therefore, this should be a good approach to include in other studies as well. It was noticed that each serological marker perceived a different outcome, resulting in a broad range of half-lives from approximately six month up to more than two years. Till recently not many studies have been capable of given an actual number to the Ab half-lives, except for Wipasa *et al.* [29] (7.6 years PfMSP1, 10.4 years PfAMA1), Fowkes *et al.* [25] (7.6 years PfAMA1, 4 years PvAMA1), Drakeley *et al.* [35] (49.8 years PvMSP1) and Kinyanjui *et al.* [27](clearance of approximately 6-10 days MSP1 and AMA1). There is still a major

drawback, because, the used statistical approaches are only capable of measuring each antigen separately. This still does not benefit from the advantage that the Luminex can screen up to 100 different Ags simultaneously [19]. Another drawback is that age, due to maturation of the immune system influences, the Ab-levels that can affect the force of infection [46]. Although age have been taken into account in the models used in **Chapter 6**, it remains difficult to interpret. The length of malaria exposure and differences in body built play a role, as well as that young children experience more variations in Ab-levels than adults [47]. The latter is due to a more rapid decay in Ab-levels in young children in absence of reinfection [48]. Therefore, further research is required.

Reinforcing the use of the multiplex immunoassay, by searching for a perfect combination of Ags that exhibit the same biological activity and could strengthen the outcomes when analysing them together was already attempted by Jepsen *et al.* [49]. They used the discrimination index, a measure of quality when the purpose is to produce a spread of scores. To obtain the discrimination index, they combined several Ags into one multiple regression model, resulting in an increase in sensitivity of the multiplex immunoassay, while converting the readouts off all the Ags into one single index for positivity. The best discrimination index was perceived when combining Pf.GLURP.R2, Pf.MSP3, PfAMA1 and Pf.GLURP.R0. In addition, Sturrock *et al.* [50] also stated that the conventional serological marker used so far have not been capable of distinguishing recent from past exposure. By this means they suggest to seek for new serological markers that are competent to differentiate exposure between several time scales. To date, researchers are still trying to find the perfect serological makers, and therefore more in-depth analyses are required.

4. Suitability of the multiplex immunoassay to detect hotspots/clusters/pockets and hotpops

In the recent years, various researchers found that serological markers are particularly suited to areas with low malaria transmission intensity that follow a seasonal transmission pattern [36]. Therefore, these markers might have potential for the identification of risk factors in malaria hotspots [51], or in case of **Chapter 7** malaria pockets. A malaria pocket refers to an area that has a size in between a hotspot (± 1 km²) and a foci (> than 1 province) and that showing higher malariometric indices from the surrounding [52, 53]. In **Chapter 7** was seen that malaria pockets perceived with serology for *P. falciparum* were similar compared to PCR prevalence data, however differences were obtained for *P. vivax.* These differences can be explained on the basis of the long half-life of the used *P. vivax* serological markers, and that the different

malaria pocket found in the PCR-prevalence data showed to be the pocket with the lowest relative risk. The opposite was observed between the serological based malaria pockets and the Incidence based pockets. The different pockets occurred in communities based around the capital city ('Ban Lung') of the Ratanakiri province. This is explainable as Incidence data is based on symptomatic cases, whereas the serology was based on mainly asymptomatic cases. It is possible that the amount of symptomatic cases was so low, that Incidence data still perceive malaria pockets. However, these small amounts of symptomatic cases most likely did not have an influence on the mean Ab-levels, resulting in no observed malaria pockets. Another explanation could be the movement of people living in the communities around 'Ban Lung' to the more remote arear. These movements were observed by other studies [52, 54, 55] as well, of whom it was notified that people show mobility between villages, regions or even cross the borders. These studies, and in particular a study performed by Parker *et al.* [55], suggested that these movements need more investigation, and that it is worthwhile to include the movement in a risk factor analysis. A last reason that differences in malaria pockets between the different approaches appear in **Chapter 7** could suggest that some geographical areas are specified as being species-specific. The primary vectors in Ratanakiri are *An. dirus* and *An. minimus*, however, it is possible that these areas contain other ecological conditions than these vectors require. This brings to mind that different malaria vectors could be present, and might explain why serology missed these pockets [56].

Another important aspect in defining spatial patterns in malaria exposure is the stability of malaria pockets over time. Several studies have observed stable hotspots/cluster/pockets over time by using spatial scan statistics on data from Kenya, Africa. Bejon *et al.* [57] focused on perceiving stable hotspots over seven years. Ernst *et al.* [58] could predict spatial clusters over a period of four years. Bousema *et al.* [59] did the same approach over three years and a last study performed by Mosha *et al.* [60] on data from Tanzania over two consecutive years found that hotspots of PfAMA1 are stable over a longer time span. These findings were confirmed by the PvAMA1 and Pf.GLURP.R2 serological markers in **Chapter 7**. The main pocket found for PvAMA1 seemed to be consistent over the two years, whereas Pf.GLURP.R2 showed a stable pocket that slightly decreased over time. All these studies support each other showing spatial scan statistics can perceive hotspots/clusters/pockets based on serological measurements and that they remain stable for at least one year.

Numerous of risk factors can lead to an increased risk of malaria infection in some areas compared to other areas. Here, you should think of human behaviour in which

they move between villages or work in the forested areas, genetic or environmental factors. From the study conducted in **Chapter 7**, only certain risk factors were investigated in order to define a pattern among the observed malaria pockets. From **Chapter 7** could be deducted that age, in particular the older age groups (>16), and overnight stay in plot huts play an important role for all *P. falciparum* Ags, as well as that an interaction was observed between age and the ethnic minority. The latter was not surprising, as the study area was selected based on the proximity to the border with Vietnam, the high presence of the indigenous groups (ethnic minorities) [52]. These people make a living by subsistence slash-and-burn farming on plot huts located near or inside the forest. Therefore, during the rainy season they leave the villages and engage in the forest activities [61]. With this in mind, it would be expected that the ethnic minority and the overnight stay in plot huts would play an important role as a risk factor, as well as the age groups reflecting cumulative exposure. *P. vivax* Ags on the other hand did not show any striking risk factors, besides for age. The fact that age can play a role as risk factor in low endemic settings was observed by previous studies as well [55, 62–65] based on PCR prevalence data, and by Mosha *et al.* [66] based on serology. Mosha *et al.* [66] suggest that children between 5 and 15 years have higher odds than children under five, because they tend to play more outdoors in the late evening and night. On the other hand, as it is observed that entire families go into the field and sleep in plot huts even the children under five [62, 63] you actually would not expect such risk differences between the age groups. Even though several studies do declare to have an answer on the increased risk in the older age as observed in **Chapter 7** as well, the most logical solution is that this is a reflection of the increased Ab-levels due to recurrent infections [51, 67].

As described above in **Chapter 7** is observed that antigens Pf.GLURP.R2, Pf.MSP1.19, CSP and PvAMA1 are able to detect recent infection. This was in particular visible with Pf.GLURP.R2 that showed a marked shrinkage of the malaria pockets over time, signifying that these serological markers would assist very well in control and elimination programmes. In both studies performed in **Chapter 7** and **Chapter 5** (intervention trial) it is witnessed that serology is able to confirm the outcomes perceived with PCR-prevalence and incidence data rates. This confirms that serology can make a major contribution in evaluation short term interventions for malaria elimination, and in the understanding of local malaria epidemiology in low endemic countries. This is confirmed by a study performed by Sylla *et al.* [68] that focused on Falciparum serological markers AMA1 and CSP in an area with declining malaria transmission. An increase with age and active malaria infection in the studied area and residences were experienced, suggesting that these serological markers have potential

as epidemiological purposes in intervention trials. In addition, more studies agree on this assumption that serosurveillance tools and strategies contribute in malaria control and elimination [43]. They attribute this on the advantage of serology in indicating malaria transmission at various points in the parasite life cycle. This is fortified when various metrics (Incidence data rates, PCR-prevalence and serology) related to the mosquito, parasite and human are used in parallel with each other [69–71]. To this end the development achieved in serological measurements are important for both (ongoing) malaria surveillance and the evaluation of malaria intervention and control programmes [69]. This will benefit especially when more attention will be paid on the *P. vivax* serological markers.

5 Anopheles gambiae salivary protein gSG6 antigens

SALIV 1 and SALIV2 Ags are derived from *An. gambiae* salivary gland 6 (gSG6). These Ags might serve as a good indicator of exposure to vector bites [15] and have been described as being useful for the evaluation of the intensity of human *Anopheles* contact [72]. These Ags were chosen to evaluate the immune responses against the mosquito Ags, as these are the only salivary gland Ags available. When observing the results of both Ags throughout all studies performed in this PhD thesis, very low MFI signals have been perceived. Nonetheless, SALIV 2 showed slightly higher Ab-responses compared to SALIV1. The low Ab-responses in Cambodian malaria patients are not surprising, as these Ags are developed from the genome of an African vector *An. gambiae.* Furthermore, SALIV2 showed better results in the half-life estimation in **Chapter 6** with a half-life of less than 1 year, compared to SALIV1 showing a half-life of more than 1 year. Moreover, in the analysis where the PCR positive and negative groups were compared to each other, these Ags showed to be capable of detecting current exposure to the vector. However, due to the low MFI values it is uncertain how reliable these results are. Therefore, it would be better to develop Ags derived from the main malaria vectors in Cambodia, namely *An. dirus* and *An. minimus* [73, 74]. The latter can be explained based on the observations of a study performed by Ambrosino *et al.* [15], who observed a strong signal for SALIV1 when tested on serum from African people, where low signals were observed in serum derived from non-African travellers. Several other studies performed on people living in malaria endemic areas that focused on the human response to salivary Ags [75, 76] showed increased IgG-Abs levels with the level of Anopheles exposure, suggesting that region specific salivary Ags can be a useful approach for identifying a marker for the risk of malaria transmission [76].

6 Differences in Ab-responses between recombinant proteins and peptides

In this thesis different recombinant proteins and peptides were included. However, in case of *P. falciparum* GLURP and *P. vivax* CSP both recombinant protein (Pf.GLURP.R2 and PvCSP chimera) and peptides (GLURP and PvVK210.CSP and PvVK247.CSP) were involved, making it possible to compare the two different types of markers to each other.

The MFI values of GLURP and Pf.GLURP.R2 are significantly higher compared to the other serological markers, with higher MFI values seen for Pf.GLURP.R2 (MFI range 14000-42000) compared to GLURP (MFI range 6500-30000). Although, both markers show to be highly antigenic [77], possibly due to recurrent infections that lead to high levels of Abs produced [78]. Besides, this can also be related to the fact that GLURP markers are present at every life-cycle stage of the *Plasmodium* parasite. For both Pf.GLURP.R2 and GLURP a half-life of < 7.5 months was observed, in which Pf.GLURP.R2 showed a half-life of 1 month shorter. Therefore, looking at these outcomes it could be stated that the recombinant protein Pf.GLURP.R2 showed slightly better results than the peptide GLURP, suggesting that using a recombinant protein in seroepidemiological studies provide better results.

Peptides PvVK247.CSP and PvVK210.CSP and recombinant protein PvCSP showed very low Ab-levels, as well as no differences between the PCR positive and negative populations. However, higher MFI values were expected, as it is shown that these Ags are present in the Anopheline vector mosquito in Southeast Asia and Indonesia [29, 79]. In the half-life estimation for markers PvVK247.CSP, PvVK210.CSP and recombinant protein PvCSP, half-lives of respectively 777, 1012 and 1095 days were observed, suggesting that the peptides might perform better than the recombinant protein PvCSP.

Overall, the differences between the recombinant proteins and their linear peptides in this study are not really noticeable. Better, when looking at the recombinant proteins and peptides belonging to different malaria parasites and life-cycles stages it appears that 3 recombinant proteins (Pf.GLURP.R2, LSA3.RE, Pf.MSP1.19) and 4 linear peptides (CSP, GLURP, SR11.1 and SALSA2) perceived the best results in reflecting current malaria exposure, throughout the entire study. Therefore, it would be of great value to perform more studies in which recombinant proteins and linear peptides from the same parasite and life cycle are compared to each other. One of the main reasons to substantiate the importance of such study is that recombinant proteins are produced using a plasmid vector [24]. This is not a very standardised method, because

differences between the same recombinant proteins can occur when produced in a different vector or different lab. The advantage, however, is that these recombinant proteins have a folded structure. This structure can induce a strong Ab-response [80]. Peptides, on the other hand, are smaller, represent only the epitope necessary for the Ab to bind and are easier to produce.

7 General conclusion

The current PhD carried out in the low endemic province of Ratanakiri, Cambodia was the first that successfully implemented a multiplex immunoassay for seroepidemiological purposes on three different *Plasmodium* species. The assay proved to be a reliable rapid test in measuring a large variety of serological markers at once with a high reproducibility. As a result the use of different serological markers pointing at different life-cycle stages strengthened the results compared to IFAT. During the course of this thesis there has been progress in evaluating short term interventions for malaria elimination supported by Pf.GLURP.R2 (Ab half-life ~6 months) showing shrinking malaria pockets over time. This was seen for Pf.MSP1.19, CSP and PvAMA1 to a lesser extent as well. To this end, it is suggested that prevention measures towards malaria elimination benefit from serology in monitoring (ongoing) malaria transmission.

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FUTURE PERSPECTIVES

9

During the past years many different approaches that made an attempt in eliminating malaria have been developed on large scale and facilitated the widespread emergence of malaria resistance. Mention may be made to the widespread of insecticidal nets, indoor residual spraying, topical repellents, accessible diagnostic tools, available treatment, and epidemiological measurements in especially low-endemic areas. Unfortunately, the ability to efficiently targeting the remaining malaria reservoirs in these low endemic areas, are to the utmost extent still undetected by the standard diagnostic tools. Appropriate scale-up and roll-out strategies should be developed to guarantee an effective reduction of malaria transmission. One of the major steps is improving the surveillance tools, as they can provide key knowledge leading to the most effective way to eliminate malaria. In this thesis the emphasis lies on the use of serology in epidemiology. Serology has repeatedly proven its additional value parallel to PCR in identifying current and past malaria exposure, determining risk factors for malaria, perceiving spatial malaria pockets and to observe a protection scale in targeting asymptomatic and symptomatic carriers. It should be noted that to date the multiplex assay is most frequently compared to ELISA and PCR. However, IFAT is the only validated 'gold standard' with which the multiplex assay should be compared to. Unfortunately, this is only reliable for *P. falciparum*, suggesting that future development towards a true 'gold standard' for all *Plasmodium* species is required.

Many researchers encounter problems with the amount of positive and negative control sera needed to perform multiple immunoassays within large trials. In many cases no control sera is left at the end of such trials, and therefore, these test cannot be performed under similar conditions later in time. For that reason, it might be useful that a control serum becomes standardized and commercially available. Another possibility might be the use of custom monoclonal Abs that can be recognized by recombinant proteins or linear peptides.

In a short period of time, many progresses have been made in the use of serological markers. Several studies, including this thesis, found promising *P. falciparum* serological markers that are most likely to reflect current and past malaria exposure. However, this is still in its infancy. On the other hand serological markers against *P. vivax* only reflect long term exposure, as only long Ab half-lives are perceived. Therefore, validating more serological markers with short half-lives will benefit in especially countries that aim towards malaria elimination and where *P. vivax* appears to be the predominant species. To extend the panel of existing Ags, it might be useful to create more specific markers by focusing on the strains that circulate in a particular

area. For example, by creating salivary gland proteins specific for *An. dirus* and *An. minimus*, as these are the main vectors present in Cambodia.

In case of spatial clustering several serological markers were able to detect malaria pockets in Ratanakiri, Cambodia. These included 3 *P. falciparum* and 1 *P. vivax* Ag. However, to increase the effectiveness of the serological markers it will be useful to validate them in several different endemic setting as well.

A final critical issue is serological modelling. There is already a point reached in which the multiplex assay performs very well in screening multiple Ags simultaneously, however serological data analyses are still performed Ag per Ag. As long as researchers cannot analyse serological markers simultaneously, without the use of a threshold model, serology will remain in the developmental process. At present, such models are already designed for viruses and could be interesting for malaria in the future as well.

SUMMARY

Malaria, a vector-borne disease caused by a *Plasmodium* parasite, is one of the most deadly infectious diseases. Despite large progress in malaria control, malaria remains an important public health concern. For countries heading to elimination, evaluation and implementation of additional malaria control tools is crucial. However, follow-up of malaria prevalence and incidence using classical malariometric tools becomes challenging in low malaria transmission settings. Seroprevalence offers an alternative for evaluating malaria transmission as anti-malaria antibodies can be sufficiently long-lived to reflect current, recent or past malaria exposure. Serological markers for exposure to different *Plasmodium* species exist and have been recently used in a multiplex assay based on the Luminex-technology. However, interpretation of the assay results requires caution and need to take the half-life of specific antibodies into consideration.

Therefore, the **main aim** of this PhD thesis is **to see if a multiplex immunoassay has additional value in monitoring (ongoing) malaria transmission at community level, and thereby detecting changes in malaria transmission intensity due to seasonality or interventions in a hypo-endemic transmission setting.**

Our **specific objectives** were:

1. To **implement** a multiplex immunoassay and **document** its value as compared to other serological tests
    a. By reviewing the multiplex serological technique to the immunofluorescence assay technique
    b. By implementing the multiplex immunoassay for detection of antibodies against antigens of different life stages and malaria parasites.
    c. By documenting the half-life of the used serological markers by analyzing sequential and cohort samples from individuals in Ratanakiri province, Cambodia.
2. To **validate** multiple serological markers for **estimating malaria transmission**
    a. By evaluating changes in malaria transmission due to an intervention trial.
    b. By identifying geographical clusters of malaria transmission.

In this thesis we have **successfully implemented** the multiplex serological assay. The method proved to be **valuable for the detection of anti-malarial antibodies** in human blood samples in a region with a relatively low prevalence. The assay can **include multiple peptides and recombinant proteins** at the same time, making it more economic, faster and more standardized compared to the classical techniques.

Moreover, this thesis has shed some light on which antibodies are **good markers for current malaria exposure**. The interpretation of the decrease of antibody intensity made it possible to drawn some conclusions on the half-life of the antibodies. The half-lives range from approximately six months to more than two years, depending on the serological marker used. Four out of the 20 serological markers screened might have potential to be reflective for recent exposure in low endemic areas. **Further validation** of these serological markers by identifying geographical clusters of malaria transmission proved the **added value** of some of these markers to reflect current exposure.

This study was part of a cluster randomized trial based in Cambodia that is on the verge of eliminating malaria. During this cluster randomized trial, epidemiological surveys were organized to study the efficacy of repellents as added control measure to long lasting insecticidal nets. Two study arms (a control and intervention arm) were compared based on PCR as a primary outcome and on serology and malaria incidence as secondary outcomes. The comparison between the control and intervention arm did not show any significant difference in seroprevalence for all antigens, which confirms the analyses performed earlier on PCR prevalence and malaria incidence.

In **conclusion** Pf.GLURP.R2 out of all different serological markers tested throughout all chapters, showed to be most informative for current infection. However, the other antigens should certainly not be ruled out, as they probably reflects transmission in the former past for which no PCR-prevalence data may be available. Further in-depth research on the serological markers and statistical modeling is required to obtain better information on ongoing malaria transmission.

NEDERLANDSE SAMENVATTING

Malaria is een van de meest dodelijke infectieziekten overgedragen door muggen en wordt veroorzaakt door een protozoa van het genus *Plasmodium*. Hoewel er al grote vooruitgang is geboekt in de bestrijding tegen malaria, vormt malaria nog altijd een belangrijk gezondheidsrisico. Voor de landen die zich in een eliminatie fase van malaria bevinden is het noodzakelijk dat er nieuwe tools voor malaria controle geëvalueerd en geïmplementeerd worden. Hierbij moet men er rekening mee houden dat het opvolgen van malaria prevalentie, met behulp van de klassieke tools in landen met een lage malaria transmissie, een uitdaging kan zijn. In dit geval biedt seroprevalentie een goed alternatief om de transmissie intensiteit in laag-endemische gebieden te kunnen evalueren, omdat anti-malaria antilichamen langer in het bloed blijven dan parasieten. Serologische markers zijn zeer recentelijk toegepast in een multiplex assay gebaseerd op de Luminex technology. Hoewel deze techniek succesvol geïmplementeerd werd, is gebleken dat het interpreteren van deze resultaten niet zo eenvoudig is.

Het **doel** van deze doctoraatsthesis is daarom om **te zien of het gebruik van een multiplex immunoassay een toegevoegde waarde heeft in het controleren van (lopende) malariatransmissie op populatie niveau, en daarbij het aantonen van veranderingen in de malaria transmissie intensiteit ten gevolge van seizoensgebonden invloeden of interventiestudies in een hypo-endemisch transmissie gebied.**

De **specifieke doelstellingen** waren:

1. Het **implementeren** en **evalueren** van een multiplex immunoassay ten opzichte van andere serologisch testen
    a. Door het vergelijken van de multiplex serologische techniek met de immunofluorescence assay techniek
    b. Door het implementeren van een multiplex immunoassay voor de detectie van antilichamen tegen antigenen van verschillende levens stadia en malaria parasieten
    c. Door het documenteren van de halfwaardetijden van de gebruikte serologische markers, door gebruik te maken van sequentiële en cohort stalen van individuen uit Ratanakiri, Cambodia
2. Het **valideren** van de serologische markers om **malaria transmissie** aan te kunnen tonen
    a. Door het evalueren van veranderingen in de malaria transmissie ten gevolge van een interventie studie
    b. Door het identificeren van geografische gebieden van malaria transmissie

In deze thesis hebben we de multiplex serologische test **succesvol geïmplementeerd**. Deze techniek heeft bewezen zeer **waardevol te zijn in het detecteren van anti-malaria antilichamen** in menselijke bloedstalen in een gebied met een lage malaria prevalentie. Deze test is in staat **meerdere peptiden en recombinante proteïnen** gelijktijdig te testen. Dat maakt deze test economischer, sneller en meer gestandaardiseerd dan de klassieke methoden. Bovendien, heeft deze thesis meer inzicht gegeven in de rol die deze serologische markers spelen in het **aantonen van recente blootstelling** aan malaria. Het interpreteren van een dalende antilichaam-intensiteit heeft het mogelijk gemaakt om de halfwaardetijd van deze antilichamen te kunnen berekenen. Deze varieert van ongeveer zes maanden tot meer dan twee jaar en is afhankelijk van de serologische marker. Het is gebleken dat vier van de 20 serologische markers, die geanalyseerd werden, in staat kunnen zijn om recente blootstelling aan malaria in laag-endemische gebieden aan te tonen. Tijdens de **verdere validatie** van deze markers in het identificeren van geografische gebieden van malaria transmissie, is gebleken dat ze een **toegevoegde waarde** hebben in het aantonen van recent contact.

Deze studie maakte deel uit van een cluster gerandomiseerde studie gevestigd in Cambodia, een gebied dat zich in de pre-eliminatie fase bevindt. Tijdens deze studie werd er een epidemiologisch onderzoek opgesteld om het gebruik van veilige en doeltreffende muggen werende middelen op malaria transmissie, bovenop het gebruik van geïmpregneerde bednetten te testen. Tijdens het verloop van deze studie weren twee studie armen (een controle en een interventie arm) vergeleken op basis van PCR als primaire uitkomst en serologie en malaria incidentie als secundaire uitkomsten. Er werd geen significant verschil in de seroprevalentie tussen de controle en interventie arm aangetoond, dat de uitkomsten van de PCR prevalentie en malaria incidentie bevestigd.

Als **conclusie** kunnen we stellen dat Pf.GLURP.R2 vergeleken met alle 20 antigenen het meest informatief is voor het aantonen van huidige infectie. Dat wil niet zeggen dat we de overige antigenen moeten uitsluiten, omdat deze markers wel eens een weerspiegeling kunnen zijn van een transmissie in het verdere verleden. Helaas zijn er alleen geen vroegere PCR-prevalentie gegevens beschikbaar om dit verder te onderzoeken. Verder onderzoek naar serologische makers en betere statistische modellen is nodig om meer informatie betreffende malaria transmissie te verkrijgen.

CONTRIBUTIONS TO THIS DISSERTATION

Sample collection of the survey was performed by the CNM team (National Center for Parasitology, Entomology and Malaria Control), the ITM Antwerp team and the Institut Pasteur du Cambodge team in Ratanakiri province in Cambodia. The study design and literature research has been performed at the ITM by KK, LD and MC. Protocols were written by KK, based on protocols from Institut Pasteur de Madagascar, verified by LC and validated by DM. Laboratorial work for the performance of the assay was performed by KK and LC, and KK executed the screening of the blood spot samples from survey 1. Laboratorial work for the analysis of the field samples from survey 2 and 4 was performed by KK, LW and SB. Selection of the pool of control sera and the high positive control dilutions was done by KK, LD and LC. Data entry and analysis of the results (made with the R-software) were performed at the ITM by KK, and verified by LD, VS and MC. The first draft of the manuscript was written by KK. All authors read and approved the final manuscript.

This work is part of a larger project with the aim of evaluating repellents as added control measure to long lasting insecticidal nets to target residual transmission in Cambodia (2012-2015). Next, this PhD project fits within the large scale research project from the 'SOFI research program' entitled 'Eco-epidemiology of asymptomatic malaria hotspots, a prerequisite for malaria elimination? These projects are implemented together with the institute Pasteur du Cambodge and the Cambodian National Malaria Control Program. This work was funded by the Bill & Melinda Gates Foundation under the Global Health Grant number OPP1032354. KK was supported by the VLIRUOS and the association of *Les Amis des Instituts Pasteur à Bruxelles.*

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Kerkhof K, Canier L, Kim S, Heng S, Sochantha T, Sovannaroth S, et al.: **Implementation and application of a multiplex assay to detect malaria-specific antibodies: A promising tool for assessing malaria transmission in Southeast Asian pre-elimination areas.** *Malar J* 2015, **14**:338.

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Maketa V, **Kerkhof K,** Mavoko H.M, Nabasumba C, Durnez L, Lutumba P, et al. **Intra- and inter-individual variability between Ab-levels in children living in holo-endemic settings, using a multiplex assay.** [In progress]

ABOUT THE AUTHOR

The author, **Karen Kerkhof**, was born on January 11, 1985 in Utrecht, the Netherlands. She took her primary education at the 'Anne Frank School' in Bunnik and in 2006 she received her VWO high school diploma at the 'ROC midden Nederland' in Zeist. That same year she studied at the Academy of Natural Medicine in Hilversum where she earned her propaedeutic diploma in Chinese medicine and acupuncture. The following summer she traveled to Guatemala to become a volunteer at a disabled people hospital for three months, where she gained her special interest in tropical diseases. Thereafter, she started with Biomedical Sciences at the University of Antwerp.

During her entire study career she spent a lot of time in top-level sports. In secondary school she performed field hockey, gymnastics and show dance at professional level. During her bachelor she commuted between Antwerp and the Netherlands for dance trainings in the weekends and continued field hockey during the week. In her third bachelor year she attended the European Championships for indoor field hockey in Ankara, Turkey where they conquered the first place.

During the first three years at the University of Antwerp Karen became an active member at the student society 'Biomedica' and was responsible as webmaster, scriptor and for organizing all sports activities. Subsequently she worked as co-worker in blood sampling during the summer months. Throughout the rest of the year she is a scuba diving assistant instructor or is traveling to explore the world.

In November 2012 she started her Master thesis at the 'Institute of Tropical Medicine Antwerp' (ITM) in collaboration with 'Institut Pasteur du Cambodge' (IPC). During her master thesis she received a grant from the VLIR-UOS to spent five months in

Cambodia for the laboratorial work. She performed her master thesis under the direct supervision of Dr. Lies Durnez and Prof. Dr. Marc Coosemans. During this year she has successfully implemented the multiplex bead-based immunoassay based on information from previously performed studies. In 2013 she received her MSc degree in Biomedical Sciences 'Infectious and Tropical Diseases' with distinction.

In November 2013 she greatly welcomed the opportunity to start her PhD at the University of Antwerp in collaboration with the ITM and IPC under the direct supervision of Dr. Lies Durnez and Prof. Dr. Marc Coosemans. The PhD project assignment was to document the half-life of IgG-antibody responses to serological markers by analyzing sequential samples from individuals in Ratanakiri, Cambodia, and by this looking at the sensitivity of the markers to pick up changes in malaria transmission. Useful markers will then be used for defining present and past hotspots of malaria transmission. Therefore, she spent the first six months at IPC in Cambodia, to execute more laboratorial work. In 2015 and 2016 her PhD was partly supported by the association of "Les Amis des Instituts Pasteur à Bruxelles".