The use of antimalarial antibodies to measure malaria transmission in low transmission and pre-elimination settings

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To Helen,

for showing me beauty, creativity and curiosity,

for your love, light and joy,

you will always be in my heart.

To Jan,

for showing me inspiration, compassion and loyalty,

for your passion for learning,

for all your love and support.

I, Lotus Leonie van den Hoogen, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Global declines in malaria transmission in recent years have refocused efforts towards elimination. An essential part of this effort is adequate detection of transmission patterns. However, meta-analyses have shown that microscopy detects about half of all polymerase chain reaction (PCR)-confirmed infections. To date, the performance of rapid diagnostic tests (RDTs) relative to microscopy and PCR has not been evaluated in a comprehensive meta-analysis. Therefore, the relationship between PCR, RDT and microscopy prevalence estimates in asymptomatic populations was determined using data from cross-sectional surveys in endemic settings (**Chapter 3**). Overall, RDTs detected 41% of all PCR-confirmed infections, and RDT-undetected (i.e. low-density) infections increased with age and decreasing transmission intensity.

Another approach to estimate transmission is to use malaria-specific immune responses of resident populations as a measure of exposure to infection. Antimalarial antibodies, in combination with age, reflect both historical and recent exposure. Until recently, the majority of sero-surveillance data were based on a few well-characterised antigens using enzyme-linked immunosorbent assays (ELISA). However, which antibodies most accurately reflect exposure to recent low-density infections remains largely unknown. To address this, **Chapter 4** examined antibody responses in previously naïve, controlled human malaria infections (CHMI) participants using protein microarray. Nearly all participants showed measurable antibody responses to a subset of four antigens one month post-CHMI. In addition to protein microarray, multiplex bead assays (MBAs) enable multiplex detection of antibodies. However, MBA protocols may require further adaptation in scenarios where results must be readily available to inform control and elimination policies. The precision of an adapted MBA protocol with improved throughput and ease-of-use was determined in **Chapter 5** using data collected in three large-scale transmission surveys. For some antigens, inter-plate variability seemed to increase during the third survey, possibly due to long-term storage of reagents.

Commercially available ELISAs are standardised in their production and have been used to test blood products prior to donation to reduce the risk of transfusion-transmitted malaria. However, their performance in an epidemiological context has not been investigated. In **Chapter 6**, one of five commercially available ELISAs evaluated, accurately described transmission in a low transmission and pre-elimination setting. A low-cost, high-throughput assay for which results are readily interpretable may help to directly inform control activities targeting areas with remaining transmission.

Acknowledgments

I would like to thank the many people who have helped me in making this PhD happen.

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To colleagues involved in the different projects I worked on over the course of my PhD:

To all those involved in the Malaria Zero project within and outside of Haiti, in particular Eric Rogier, Thomas Druetz, Ruth Asthon, Vena Joseph, Thomas Eisele, Alyssa Young, Karen Hamre and Michelle Chang.

To the LNSP team in Haiti: Alexandre Existe, Jacquelin Présumé, Ithamare Romilus, Gina Mondélus and Tamara Elismé.

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To Logan, for always being there for me and for reminding me what is important in life.

Statement of Contributions and Additional Publications

The work presented in this thesis was conducted primarily at the London School of Hygiene and Tropical Medicine (LSHTM) in the UK, but also at the Radboud University Medical Centre (RUMC) in Nijmegen, the Netherlands and at the Laboratoire National de Santé Publique (LNSP) in Port-au-Prince, Haiti. Below I have detailed my contribution to each of the chapters presented in this thesis as well as those of different collaborators, although this list is incomplete as there were many more people involved in this work such as field data collection teams, drivers, project managers and others.

Chapter 1

The first chapter describes details around the use antimalarial antibody metrics to measure malaria transmission patterns and platforms for antibody detection. Section 1, 2 and 5 mainly constitute original work. Section 2 presents a short overview of key malaria metrics currently available to the malaria research and surveillance field. The Tables and Figure in this section are from the MalERA (Malaria Eradication Research and development Agenda) Refresh publication summarising the results from an expert consultative panel discussion on "Characterising the reservoir and measuring transmission". I acted as rapporteur for this panel and was part of the writing committee for the resulting report published in 2017. Section 3 and 4 were published as chapters in the Springer Link Encyclopedia of Malaria. I performed the literature review and wrote the first draft of these chapters under supervision of Chris Drakeley (CD).

The malERA Refresh Consultative Panel on Characterising the Reservoir and Measuring Transmission (2017) malERA: An updated research agenda for characterising the reservoir and measuring transmission in malaria elimination and eradication. PLoS Med 14(11).

van den Hoogen L, Drakeley C (2017) Immunoepidemiology for the Evaluation of Malaria Transmission Patterns. In: Kremsner P, Krishna S (eds) Encyclopedia of Malaria. Springer, New York, NY.

van den Hoogen L, Drakeley C (2015) Malaria Diagnostic Platform, Antibody Detection. In: Hommel M, Kremsner P (eds) Encyclopedia of Malaria. Springer, New York, NY.

Chapter 2

This chapter describes the objectives of the research projects presented in this thesis.

Chapter 3

In Chapter 3, results are presented from a large-scale meta-analysis of RDT, microscopy and PCR prevalence estimate across published and unpublished data from cross-sectional surveys in endemic settings. This project was part of the Diagnostic Modelling Consortium led by Azra Ghani (Imperial College, London, UK). Lindsey Wu (LW, LSHTM) and I contributed equally to the literature review, dataset management, communication with researchers, data analyses and writing. Within the data analyses, LW focussed on Bayesian analyses of best fit relationships between cluster prevalence estimates, while I focussed on the logistic regression analyses of explanatory factors for discordance in measurements using individual-level data. LW and I wrote the first draft of the manuscript together, with support from Lucy Okell, Hannah Slater, Patrick Walker, Azra Ghani (Imperial College) and CD.

Wu L*, van den Hoogen LL*, Slater H, Walker PGT, Ghani AC, Drakeley CJ, et al. (2015) Comparison of diagnostics for the detection of asymptomatic Plasmodium falciparum infections to inform control and elimination strategies. Nature; 528:S86–93.

*Shared first author.

Chapter 4

Chapter 4 uses data from eight formerly conducted CHMI trials in previously naïve volunteers at the RUMC in Nijmegen. These samples were analysed by protein microarray to determine which antigens induce measurable antibody responses following exposure to recent low-density infections. Robert Sauerwein (RS), Jona Walk (JW), Isaie J. Reuling (IR) and Teun Bousema (TB) were involved in the design and performance of the original CHMI trials, and collection of all samples (RUMC). The antigens included on the protein microarray were designed/provided by Kevin KA Tetteh (KT, LSHTM), Linda Reiling, James G. Beeson (JB), Ross L. Coppel, Susheel K. Singh and Simon Draper. Tate Oulton (TO, LSHTM) and KT designed the protein microarray assay. I processed samples with support from TO. I also performed data analyses and wrote the first draft of the manuscript with support from JW, TO, IR, JB, TB, CD, RS and KT.

van den Hoogen LL, Walk J, Oulton T, Reuling IJ, Reiling L, Beeson JG, Coppel RL, Singh SK, Draper SJ, Bousema T, Drakeley C, Sauerwein R and Tetteh KKA (2019) Antibody Responses to Antigenic Targets of Recent Exposure Are Associated With Low-Density Parasitemia in Controlled Human Plasmodium falciparum Infections. Front. Microbiol. 9:3300.

Chapter 5

Chapter 5 describes the data collection and quality control process for antibody detection from samples collected in large-scale transmission surveys in Haiti. This work was part of the Malaria Zero (MZ) Consortium in which multiple organisations have partnered aiming to reduce and eliminate malaria in Haiti. The MZ Consortium is led by Michelle Chang (MC; Centers for Disease Control and Prevention, CDC, Atlanta, Georgia, USA) and Jean Frantz Lemoine (JFL; Ministère de la santé publique et de la population, Haiti). Colleagues at the Tulane University in New Orleans, Louisiana, USA (Thomas Druetz, TD; Ruth Ashton, RA; Thomas P. Eisele; TE), the CDC (Karen Hamre, KH; and MC) and LSHTM (CD and Gillian Stresman, GS) led survey design. Colleagues at Tulane University (TD; RA and Vena Joesph, VJ) and the CDC (KH and MC) were involved in the collection of samples in the field. Eric Rogier (ER, CDC) led the logistics of the setup of the laboratory at LNSP. I supported the set-up of the laboratory at LNSP as well as the training and supervision of the laboratory team in using the MBA, together with ER. I also supported the roll-out of cross-sectional surveys in the central and southwestern parts of Haiti (with TD, VJ, KH, MC and others).

This chapter presents the retrospective quality control of the collected antibody response data (IgG) from the first three surveys conducted in Haiti using MBA. KT provided the antigens and ER prepared bead sets. The LNSP team performed all the IgG data collection for survey samples: Jacquelin Présumé, Ithamare Romilus, Gina Mondélus and Tamara Elismé. I provided in-country and remote support, together with ER. Laboratory support and management was further provided by Alexandre Existe and Jacques Boncy. I performed dataset management, data analyses and wrote the first draft of the manuscript with support from ER and CD.

Chapter 6

In Chapter 6 commercially available ELISAs are evaluated for their application and performance in epidemiological surveys to characterise malaria transmission. CD, GS, Nuno Sepúlveda (NS, LSHTM), Alan Kitchen (AK, NHS Blood and Transplant) and Peter Chiodini (PC; Hospital for Tropical Diseases) conceived the study. I, together with CD, GS, NS, AK and PC, contributed to the grant proposal for this study. For Phase I, assay performance was assessed. Amongst other factors, this included an assessment of specificity and cross-reactivity. For these assessments, AK provided anonymised NHS Blood and Transplant donor samples. For Phase II, samples from two endemic settings were analysed: Praia, Cape Verde and Bataan, the Philippines. CD, GS and NS designed the field surveys. NS, José Moniz Fernandes (University of Cape Verde), Joana Alves and Júlio Rodrigues (National Institute of

Public Health, Cape Verde) performed sample collection in Praia. Paolo Bareng, Ralph Reyes (RR), Malou Macalinao (MM), Kimberly Fornace (KF), Fe E. Espino (FE), Jennifer Luchavez (Research Institute for Tropical Medicine, Department of Health) and GS performed sample collection in Bataan. RR, MM, KF and FE also provided information on passively collected case counts from local health facilities in Bataan.

For Phase I and II, I compiled the list of necessary laboratory consumables, planned and performed serological assays (with support from TO), performed data analyses and summarised the results. NS provided R scripts for reversible catalytic models using age-specific seroprevalence data as well as to define mixture distribution within the antibody data. I also wrote the first draft of the manuscript with support from NS and CD. The additional files to this chapter describe the epidemiological results from endemic settings using an in-house ELISA. I optimised the in-house ELISA protocol with support from Tom Hall (LSHTM) and KT, and I collected IgG responses using the optimised ELISA for the samples from Bataan and Praia.

Chapter 7

Chapter 7 consists of original work and includes a summary of the findings presented in this thesis and a discussion of the implications.

Additional publications

I also contributed to other projects that were not part of my PhD work:

1. *van den Hoogen LL, Griffin JT, Cook J, Sepulveda N, Corran P, Conway DJ, et al. (2015) Serology describes a profile of declining malaria transmission in Farafenni, The Gambia. Malar J.;14(1):416.*

2. *Zakeri S, van den Hoogen LL, Mehrizi AA, Karimi F, Raeisi A, Drakeley C (2016) Anti-malarial seroprevalence assessment during an elimination programme in Chabahar District, south-eastern Iran. Malar J.;15(1):382.*

3. *Tadesse FG, van den Hoogen L, Lanke K, Schildkraut J, Tetteh K, Aseffa A, et al. (2017) The shape of the iceberg: quantification of submicroscopic Plasmodium falciparum and Plasmodium vivax parasitaemia and gametocytaemia in five low endemic settings in Ethiopia. Malar J.;16:99.*

4. *Keffale M, Shumie G, Behaksra S, Chali W, van den Hoogen LL, et al. (2019) Serological evidence for a decline in malaria transmission following major scale up of control efforts in a setting selected for P. vivax and P. falciparum malaria elimination in Babile district, Oromia, Ethiopia. Transactions of the Royal Society of Tropical Medicine & Hygiene, accepted.*

Table of contents

List of Figures

Figure 6: The relationship between observed and predicted rapid diagnostic test (RDT) prevalence from the RDT:microscopy comparison (**a**), and the RDT:polymerase chain reaction comparison (**b**)

Chapter 4

Figure 1: Parasitemia after controlled human malaria infection over categories of cumulative parasite density (**a-c**) and associated peak parasite densities (**d**)

Figure 2: Kinetics of antimalarial antibody responses after controlled human malaria infection

Figure 3: The number of antigenic targets recognized at three time points (**a**), standardized antibody responses (**b**) and averaged standardized antibody responses (**c,d**)

Figure 4: The association between cumulative parasite density and standardized antibody responses

Supplementary Figure 1: Duplicate median fluorescence intensity measures from repeated spots on the protein microarray

Supplementary Figure 2: Antibody responses over categories of cumulative parasite density thirty days post-challenge

Additional File, Figure 1: Comparison of median fluorescence intensity (MFI) measured by protein microarray (array) and suspension bead array (SBA; i.e. multiplex bead assay) in controlled human malaria infection participants thirty days post-challenge

Chapter 5

Figure 1: Antibody reactivity profile of positive control standards of hyperimmune sera

Figure 2: Levey-Jenning charts of antibody responses in the standard of Haitian hyperimmune sera across all plates

Figure 3: Average standard curves of the positive control standard of Haitian hyperimmune sera for each survey

Figure 4: Median and interquartile range of predicted y-inflection points of standard curves per survey using the Haitian standard of hyperimmune sera

Figure 5: Transformation of antibody responses between the OneStep and Stepwise multiplex bead assay protocols for AMA-1

Supplementary Figure 1: Antibody reactivity profile of positive control standards of hyperimmune sera as well as a and negative control standard of malaria-unexposed sera

Supplementary Figure 2: Average standard curves of the 10/198 WHO positive control standard of hyperimmune sera for each survey

Supplementary Figure 3: Average standard curves of the Haitian (HP, top) and 10/198 WHO (NIBSC, bottom) positive control standard of hyperimmune sera for each survey for the *Plasmodium falciparum*, *P. vivax* and *P. malariae* 19 kDa merozoite surface protein 1

Additional File, Figure 1: Comparison of median fluorescence intensity (MFI) measures in serum (y-axes) and dried blood spots (DBS) eluate (x-axes) as recorded by the multiplex bead assay Stepwise protocol

List of Tables

Chapter 5

Table 1: Characteristics of multiplex bead assay antigen panel for three malaria transmission surveys in Haiti

Table 2: Number of samples and observations for which IgG antibody responses were successfully collected using a multiplex bead assay across three malaria transmission surveys in Haiti

Supplementary Table 1: Frequency of optimal number of parameters using 3- to 5-parameter logistic regression analyses for standard curves on all the plates across three surveys in Haiti per positive control standard of hyperimmune sera

Supplementary Table 2: Characteristics of the transformation of antibody responses between the OneStep and Stepwise multiplex bead assay protocols per antigen: parameter estimates and goodness-of-fit for best fit n-parameter logistic regression as well as Pearson's correlation coefficients for the comparison of Stepwise and transformed responses

Chapter 6

Table 1: Assay characteristics for five commercial enzyme-linked immunosorbent assays for antimalarial antibody detection (A-E) according to instruction manuals

Table 2: Costs per sample, amount of serum needed to run a sample, ease-of-use, specificity and cross-reactivity for five commercial enzyme-linked immunosorbent assays for antimalarial antibody detection (A-E)

Table 3: Logistic regression analysis of explanatory factors for seropositivity as recorded by enzyme-linked immunosorbent assay A in Praia, Cape Verde and Bataan, the Philippines

Supplementary Table 1: Standard operating procedures for five commercial enzyme-linked immunosorbent assays for antimalarial antibody detection (A-E) and composite measure of ease-of-use

Supplementary Information II, Table 1: ID (A-E) and brand names for five commercial enzyme-linked immunosorbent assays for antimalarial antibody detection

Supplementary Information III, Table 1: Seroprevalence as recorded by enzyme-linked immunosorbent assay E in Praia, Cape Verde and Bataan, the Philippines (overall and by age category)

Additional File, Table 1: Assay characteristics for five commercial (A-E) and the research-based enzyme-linked immunosorbent assays for antimalarial antibody detection according to instruction manuals

Additional File, Table 2: Costs per sample, amount of serum needed to run a sample, ease-of-use, specificity and cross-reactivity for five commercial (A-E) and the research-based enzyme-linked immunosorbent assays for antimalarial antibody detection

Additional File, Supplementary Table 1: Standard operating procedures for five commercial (A-E) and the research-based enzyme-linked immunosorbent assays for antimalarial antibody detection

Chapter 7

Table 1: Overview of published evidence of antibody responses to antigens associated with recent naturally acquired *Plasmodium falciparum* infections

Abbreviations

ABER	Annual Blood Examination Rate
ACT	Artemisinin-based Combination Therapy
AMA-1	Apical Membrane Antigen 1
AUC	Area Under the Curve
API	Annual Parasite Incidence
BSA	Bovine Serum Albumin
CBA	Cytometric Bead Array
CHMI	Controlled Human Malaria Infection
CI	Confidence Interval
cRDT	Conventional Rapid Diagnostic Test
CSP	Circumsporozoite Protein
DHS	Demographic and Health Surveys
DNA	Deoxyribonucleic Acid
EIR	Entomological Inoculation Rate
ELISA	Enzyme-Linked Immunosorbent Assay
FMM	Finite Mixture Model
GMEP	Global Malaria Eradication Programme
GR	Gametocyte Rate
gSG6	*Anopheles gambiae* Salivary Gland Protein 6
HBR	Human Biting Rate
HMM	Hidden Markov Model
HRP2	Histidine-rich Protein 2
hs-RDT	High-Sensitive Rapid Diagnostic Test
IFAT	Immunofluorescence Antibody Test
Ig	Immunoglobulin
IQR	Interquartile Range
IRS	Indoor Residual Spraying
ITN	Insecticide-Treated Net
IVTT	In Vitro Transcription and Translation
LFA	Lateral Flow Assay
LLIN	Long-Lasting Insecticide-treated Net

MalERA	Malaria Eradication Research and development Agenda
MBA	Multiplex Bead Assay
MDA	Mass Drug Administration
MFI	Median Fluorescence Intensity
MSAT	Mass Screen and Treat
MSP-1$_{19}$	19 kilodalton fragment of Merozoite Surface Protein 1
NAAT	Nuclear Acid Amplification Test
OD	Optical Density
PBS	Phosphate-Buffed Saline
PCR	Polymerase Chain Reaction
PF*Pf*	Proportion of Fevers *Plasmodium falciparum* parasitaemic
pLDH	*Plasmodium* Lactate Dehydrogenase
POC	Point-of-care
PR	Parasite Rate
qPCR	Quantitative Polymerase Chain Reaction
RDT	Rapid Diagnostic Test
SCR	Seroconversion Rate
SD	Standard Deviation
SE	Schizont Extract
SNP	Single Nucleotide Polymorphism
SOP	Standard Operating Procedure
SPR	Slide Positivity Rate
SR	Sporozoite Rate
SRR	Seroreversion Rate
SSA	Sub-Saharan Africa
USA	United States of America
WHO	World Health Organisation

Additional antigen abbreviations to those described above are detailed in **Chapter 4**, **Supplementary Table 2** and **Chapter 5**, **Table 1**.

Chapter 1: Introduction

1. Malaria Transmission: Control, Elimination & Eradication

Malaria is a vector-borne infectious disease caused by the *Plasmodium* parasite of which *P. falciparum* and *P. vivax* pose the greatest public health challenge [1]. *P. falciparum* is the deadliest of human malarias and most prevalent in sub-Saharan Africa (SSA), while *P. vivax* mainly occurs in Southeast Asian and South-American settings [1,2]. Upon inoculation by an infectious mosquito, the malaria parasite develops in the human liver and then in the blood, where its replication causes malaria disease. *Plasmodium* transmission requires sexual-stage parasites, gametocytes, in humans to be taken up by female *Anopheles* mosquitoes when they feed. After a period of parasite development, mosquitoes can then infect humans again. A break in this cycle at any point interrupts malaria transmission, if this interruption occurs in sub-regions or countries it signifies elimination or, if globally, eradication [3,4]. During the 1950s/1960s the Global Malaria Eradication Programme (GMEP) successfully eliminated the disease from many temperate regions experiencing low transmission [5]. However, it failed in higher transmission areas as the available tools were insufficient to eliminate. Elimination efforts stagnated, and in some areas reversed, due to widespread insecticide resistance as well as technical, operational and financial reasons [5–8]. The following decades saw a worldwide increase in malaria incidence until in the late 90s/early 2000s increased investment led to the discovery of long-lasting insecticide-treated nets (LLINs), rapid diagnostic tests (RDTs) and artemisinin-based combination therapies (ACTs) [9,10]. Between 2000 and 2015 these combined interventions have again resulted in impressive declines with infection prevalence halved in SSA alone [11]. In 2007, there was a renewed call to for malaria eradication and an up-to-date research agenda was defined [12,13]. However, drug and insecticide resistance due to the adaptation of the *Plasmodium* parasite and the *Anopheles* mosquito vector remain threats for control and elimination [14]. Even though more and more countries reach (pre-) elimination[1] [16], there are several settings with persistent low malaria transmission[2] despite high coverage of vector control measures and the availability of effective treatment, suggesting that novel approaches are needed for both surveillance and

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[1] Definition of pre-elimination: a phase of programme re-orientation from malaria control to elimination. Malaria programmes do not "achieve" pre-elimination status, they go through it, however an indicative milestone for finalisation and the move to the elimination phase is <1 case per 1000 population at risk per year [15].
[2] Definition of low transmission: annual parasite incidence (API) of 100-250 cases per 1000 population and prevalence of *P. falciparum*/*P. vivax* of 1-10%. Definition of very low transmission: API of <100 cases per 1000 population and a prevalence of *P. falciparum*/*P. vivax* malaria >0 but <1% [17].

interventions that will maintain and accelerate the elimination process [4,18,19]. For this, the ability to quantify malaria transmission accurately and rapidly is essential. With more countries globally reaching low or eliminating levels of malaria transmission we need to not only know how to reach zero transmission, but, intrinsically linked to this, how do we know transmission has ceased?

The following section (Section 2) will present a short overview of key malaria metrics currently available to the malaria research and surveillance field. The Tables and Figure in this section are from the MalERA (Malaria Eradication Research and development Agenda) Refresh publication summarising the results from an expert consultative panel discussion on "Characterising the reservoir and measuring transmission". I acted as rapporteur for this panel and was part of the writing committee for the resulting report published in 2017 [4]. The following two sections will firstly discuss the use of antimalarial antibodies as a metric to describe malaria transmission across populations (Section 3), and secondly the current laboratory methods for antibody detection that are used in the malaria research field (Section 4). Each section was published as a chapter in the Springer Encyclopedia of Malaria [20,21]. Any updates to the information in these two sections will be included in footnotes. The final section of the introduction (Section 5) will discuss the most recent updates to the sero-surveillance field and constitutes original work.

2. Malaria Metrics

Measures of malaria transmission can be defined at different points of the transmission cycle (**Figure 1**). Most entomological metrics (**Table 1**) are unable to quantify, or differentiate changes in, the malaria burden at low transmission due to extremely low sporozoite rates in mosquitoes. As such entomology has been identified as a major neglected area critical to elimination [14]. Human metrics (**Table 2**) are divided into passive detection (in which malaria-infected patients seek care which leads to diagnosis) and active detection (in which health care workers or researchers screen for malaria-infected patients, usually at their residence, by testing everyone willing to participate for malaria). Passively detected data are economically viable, on the basis of a health system with these services already being in place, as it provides continuous data over time [22]. However, this metric is dependent on the quality and coverage of the health system, health-seeking behaviour by local populations and the presence of symptoms during the infection. Active detection of malaria infections in a population circumvents these challenges (i.e. asymptomatic infections can be identified) and the most widely used metric is the parasite rate (PR): the proportion of people with an infection at a given point in time. However, active detection is more costly, labour-intensive and is usually a cross-sectional (i.e. single-time-point) estimate. Moreover, the sensitivity of active detection in assessing the reservoir of infection within a population is dependent on the diagnostic used. Measuring parasite infection by microscopy has been the gold standard for malaria diagnosis for more than a century. More recently nuclear acid amplification tests (NAAT; most frequently polymerase chain reaction, PCR) as well as RDT are used. PCR-based techniques are considered impractical for field surveys due to the high costs, long processing time and the lack of appropriate facilities in many endemic countries [23]. However, Okell et al. showed in 2009 that approximately 50% of all PCR-infections were missed by microscopy [24] (i.e. submicroscopic infections). Submicroscopic infections were more prevalent in adults and at low transmission [25]. Although there is limited evidence of the role of submicroscopic infections in onwards transmission to mosquitoes and countries have eliminated malaria with the use of microscopy alone, their extent at low transmission has to be considered if our aim is to eliminate [4]. So far, no studies have performed a comprehensive meta-analysis to evaluate the concordance between PCR, RDT and microscopy detection methods simultaneously in asymptomatic populations.

Furthermore, the detection of infections in cross-sectional surveys is heavily dependent on the timing of the survey, especially at low transmission, due to fluctuations in parasite rates between seasons and in parasite densities over the course of an infection. Serological metrics (discussed in detail below in Section 3), using antimalarial antibody responses in resident populations, are relatively new to the

malaria surveillance field. As antibodies represent past and therefore, cumulative, exposure, they can identify all those exposed to malaria in a single cross-section (**Figure 2**).

Figure 1. Key programmatic and research metrics across the malaria parasite transmission cycle.

NAAT, nucleic acid amplification test; RDT, rapid diagnostic test; SNP, single nucleotide polymorphism. Figure copied from [4].

Table 1: Summary of currently available entomological malaria transmission metrics. Table copied from [4].

Metric	Definition [26]	Measure of transmission	Sampling method Resolution	Discriminatory power
Entomological inoculation rate (EIR)	Number of infective bites received per person in a given unit of time, in a human population	Transmission intensity	• Human landing collection; light traps • Person or community level	• Insensitive at low transmission • Lack of standardised sampling design • Collected by malaria control programmes
Sporozoite rate (SR)	Percentage of female *Anopheles* mosquitoes with sporozoites in the salivary glands	Risk of infection	• Human landing catch; baited traps; gravid traps • Community level	• Insensitive at low transmission
Human biting rate (HBR)	Average number of mosquito bites received by a host in a unit time, specified according to host and mosquito species	Risk of exposure	• Human landing collection • Person or community level	• Allows determination of the primary vector
Vectorial capacity	Rate at which given vector population generates new infections caused by a currently infectious human case	Efficiency of transmission	• Derived from human biting rate, parasite inoculation period, mosquito to human density and mosquito survival	• Measures potential not actual rate of transmission – includes no parasitological information • Sensitive to changes in mosquito survival and biting behaviour, but may not translate to significant change in human incidence • Can be useful when infection rates are low and mosquito sampling difficult

Table 2: Summary of currently available malaria transmission metrics in humans. *No WHO definition available. Table copied from [4].

Metric	Definition [26]	Measure of transmission	Method	Discriminatory power
Annual blood examination rate (ABER)	The number of people receiving a parasitological test for malaria per unit population per year	Level of diagnostic monitoring activity	Microscopy or RDT	• Dependent on health system provision
Case, confirmed	Malaria case (or infection) in which the parasite has been detected in a diagnostic test	Current transmission or incidence if data collection is repeated or routine	Microscopy or RDT positive	• Insensitive at low transmission; saturates at high transmission • Underestimates due to system inadequacies and poor health-seeking behaviour
Case, fever	The occurrence of fever (current or recent) in a person	Current transmission or incidence if data collection is repeated or routine	Reported or observed fever	• Overestimates malaria infection
Proportion of fevers parasitaemic (PF*Pf*)*	Proportion of fever cases found to be positive for *Plasmodium*	Current transmission or incidence if data collection is repeated or routine	Microscopy; RDT; NAAT	• Depends on diagnostic sensitivity • Insensitive at low transmission
Slide positivity rate (SPR)	Proportion of blood smears found to be positive for *Plasmodium* among all blood smears examined	Current transmission or incidence if data collection is repeated or routine	Microscopy	• Depends on ABER • Insensitive at low transmission
RDT positivity rate (RDT-PR)	Proportion of positive results among all RDTs performed	Current transmission or incidence if data collection is repeated or routine	RDT	• Depends on RDT sensitivity • Insensitive at low transmission
Parasite rate (PR)	Proportion of the population found to carry asexual blood-stage parasites	Current transmission or incidence if data collection is repeated or routine	Microscopy; RDT; NAAT	• Depends on diagnostic sensitivity • Insensitive at low transmission
Gametocyte rate (GR)	Percentage of individuals in a defined population in whom sexual forms of malaria parasites have been detected	Potentially infectious human population	Microscopy; NAAT	• Depends on diagnostic sensitivity • Insensitive at low transmission

Population surveyed for parasite rate every half year

8%   0%   0%   4%   16%   4%

Serology survey will detect antibodies in all those exposed

32%
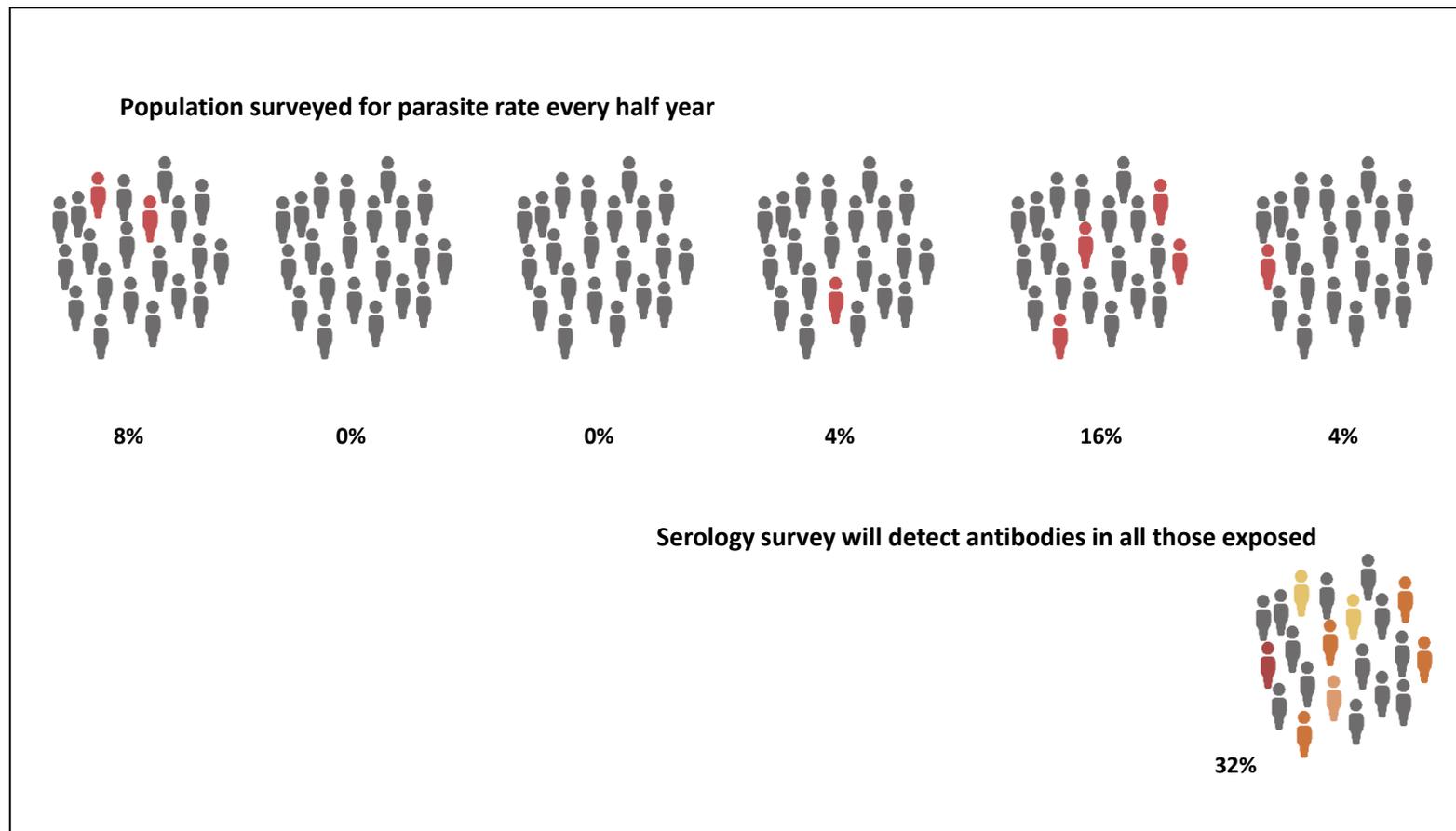
Figure 2: Schematic of the information collected in six cross-sectional surveys assessing the parasite rate compared to one cross-sectional survey assessing antibody responses. For parasite rate surveys: grey people are parasite negative and red people are parasite positive. For the serological survey: grey people are antibody negative, while the shading of colours is indicative of antibody titre (those more recently infected have higher antibody titres represented in darker colours).

3. Immunoepidemiology for the Evaluation of Malaria Transmission Patterns

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SECTION A – Student Details

Student	Lotus Leonie van den Hoogen
Principal Supervisor	Chris Drakeley
Thesis Title	The use of antimalarial antibodies to measure transmission in low transmission and pre-elimination settings

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?	Springer Link		
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31

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Immunoepidemiology for the Evaluation of Malaria Transmission Patterns

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Background

Measurement of malaria transmission patterns is important to enable better targeting of malaria control strategies as well as to assess the impact of these strategies after implementation. In addition, long-term monitoring of transmission can play a key role in the planning of a country's health expenditure in relation to current transmission dynamics. Measurements are also vital for research studies aimed at evaluating exposure to infection.

The universal measure of the capacity of a disease to spread in a population is the basic reproduction number (R_0), which represents the average number of infections one infected individual generates over the course of the infectious period in a susceptible population. R_0 is difficult to measure practically in vector-borne diseases – such as malaria – because of the highly mobile nature of the vectors, the high proportion of asymptomatic infections in certain settings that are missed by routine surveillance, and the long incubation period involved in both the human and the mosquito. Alternative metrics to measure malaria transmission intensity are the parasite prevalence in humans (PR), the entomological inoculation rate (EIR), and clinical incidence. However, each of these measures have limitations (Tusting et al. 2014), particularly in areas of low malaria transmission, and alternative or additional metrics would be advantageous.

One approach to estimate transmission is to use the malaria-specific immune responses of resident populations as a measure of exposure to infection. The most convenient form is the measurement of antimalarial antibodies. These serological data are increasingly collected in malaria research due to their field applicability, low costs, and the fact that they allow for rapid assessment of recent trends in malaria transmission intensity (Corran et al. 2007). Antimalarial antibodies reflect the history of exposure to malaria infections and can therefore provide information about the historical patterns of malaria transmission in a population. The approach has advantages at low transmission levels, where conventional methods have limited sensitivity. Furthermore, serological measures are less prone to fluctuations in parasite density over the course of an infection and over seasonal changes in transmission. In this chapter, we will discuss descriptive metrics using serological data, their applications in malaria research, as well as future directions.

Descriptive Metrics Using Malarial Serological Data

Several methods are available to measure antimalarial antibodies such as the immunofluorescence antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA). These are discussed in detail in Chapter ► "Malaria Diagnostic Platforms; Antibody Detection." Antibodies can be detected to whole *Plasmodium* parasite extract, or specific recombinant antigens or peptides. Once antibodies have been detected, the results can be used as continuous antibody responses or can be re-categorized as positive or negative.

Seroprevalence

Antibody responses can be classified dichotomously as positive or negative, and there are several ways to define this classification. The classic method is to use antibody responses of negative (i.e., nonexposed) individuals to define a cutoff for positivity. Alternatively, assuming there are seronegative individuals in the population of interest, a finite mixture model can be used to identify two populations (antibody negative and antibody positive) within the data. Generally, in low transmission areas, the mixture model is a robust method as there are more seronegative individuals, but at high transmission levels, the "negative" population could include "low response" positives. Therefore, a cutoff based on antibody responses from nonexposed individuals may be more suitable at higher transmission intensities. Defining a cutoff is somewhat arbitrary and results in a loss of information regarding the strength of an antibody response; however, seroprevalence can be a useful measure when combined with population age data. Alternative methods have been proposed, for example, providing a probability per sample that the measured response is above background rather than classifying samples as positive or negative (Irion et al. 2002). However, since serological metrics are generally used as a population-level metric, this method has not been as widely applied in malaria literature to date.

When a cutoff is defined, the seroprevalence can be calculated as the proportion of seropositive individuals in a population. Seroprevalence increases with age representing repeated exposure to malaria infections over time. However, while analyzing malaria serological data, antibody responses from infants less than 1 year old are normally excluded due to the possible transfer of maternally derived antibodies. Broadly, seroprevalence of 1–5-year-olds represents recent transmission intensity in an area, while seroprevalence in adults (>15-year-olds) represents historical transmission patterns. Furthermore, in combination with age, seroprevalence can be used to assess the force of infection (seroconversion rate).

Seroconversion Curve

The seroconversion rate (SCR) and seroreversion rate (SRR) can be calculated fitting a simple reversible catalytic model to seroprevalence and age data using maximum likelihood methods (Williams and Dye 1994). The SCR reflects the rate at which individuals become seropositive and is an indication of the force of infection in a population, while the SRR reflects the rate with which antimalarial antibodies are lost.[3] A high SCR results in a steep seroconversion curve, thus is an indicator of malaria transmission intensity. In high transmission areas, seroprevalence is saturated at a young age due to the high levels of exposure. In contrast, in low transmission areas, curves are generally less steep, with seroprevalence increasing more gradually with age due to the less frequent exposure events (Fig. 1a, b).

Examining seroconversion curves can also inform whether there have been historical changes in transmission intensity. A pronounced change in malaria transmission due to an intervention will be reflected as lower antibody prevalence in those born since the intervention was implemented. This results in a change in the serological profile with children showing lower antibody densities. In this situation, a model allowing for more than one SCR in the population can be fitted to the age seroprevalence data (Fig. 1c) (Corran et al. 2007). Several factors are associated with the likelihood of detecting a change in transmission such as the level of endemicity, the extent of the change, the time between the intervention and sampling, and

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[3] The use of "antibody loss" here refers to "reversion from seropositive to seronegative" (i.e. the dichotomous values) rather than antibody decay (i.e. loss of antibody density over time).
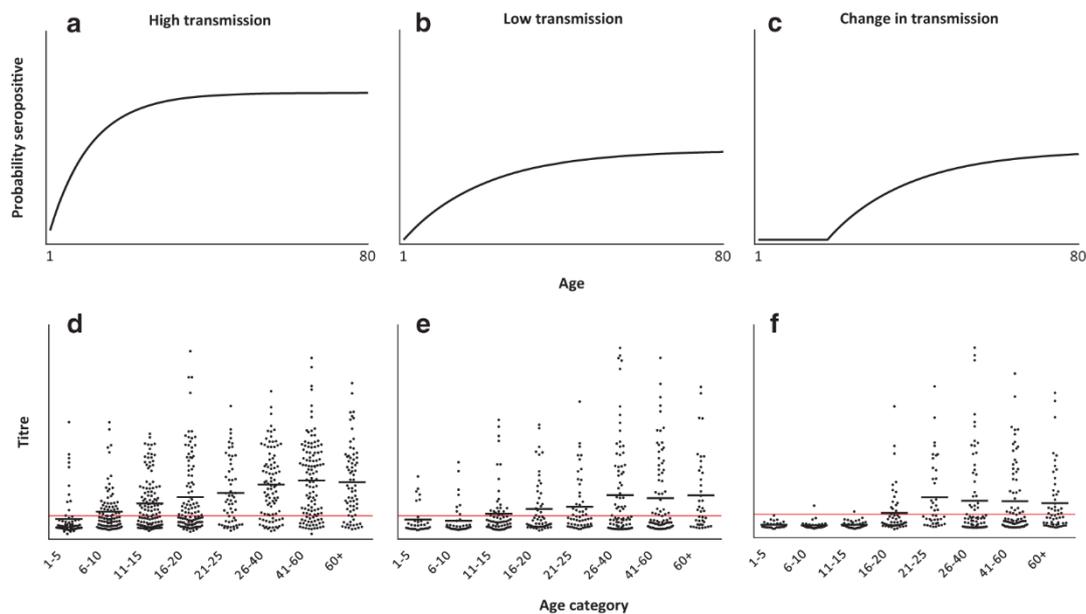
Immunoepidemiology for the Evaluation of Malaria Transmission Patterns, Fig. 1 Examples of seroconversion curves and titer distribution plots by transmission intensity. Examples of seroconversion curves (**a**–**c**) and scatter dot plots of antibody titer by age category (**d**–**f**) for high transmission (**a**, **d**), low transmission (**b**, **e**), and a change in transmission (**c**, **f**). *Dots* in figures (**d**–**f**) represent individual observations of titer, while *black horizontal lines* represent the mean titer for each age category and *red horizontal lines* represent thresholds for seropositivity

the sample size. Prior information about the timing of a possible change point could help improve the power of research studies in accurately identifying a change in transmission if correct sampling approaches are used around these age groups (Sepúlveda et al. 2015).

Seroreversion Rate

In addition to the reversible catalytic model, a superinfection model which specifically allows for prolonged periods of infection due to repeated exposure at high transmission areas can be applied (Bosomprah 2014). This model allows for an antibody boosting scenario in people who are already seropositive, thus allowing for movement between multiple states (from seronegative to multiple – superinfected – seropositive states). This model tends to result in more realistic estimations of the SRR.[4]

For the purpose of analysis, the SRR can be fixed when assessing the SCR of several surveys from the same area simultaneously, in order to be able to compare the SCRs better. However,

different rates of antimalarial antibody decay might be expected in relation to age, the type of antigen, and transmission intensity itself. The SRR described in most studies do not take different subpopulation characteristics into account, and are quite low, representing a long, and possibly unrealistic time for antibodies to decay. Methods would be improved by focusing on evaluating the SRR in relation to age and transmission intensity, in order to improve SCR estimates, but also because SRR will become important in its own right as countries attempt to eliminate malaria.

Antibody Density

As well as using binary antibody responses to define seroprevalence, antibody densities – or titers – can be used in their continuous form (Fig. 1d-f) and have been proven to be a sensitive tool to detect a change in malaria transmission. In the landmark Garki Project in Nigeria in the 1970s (Molineaux and Gramiccia 1980), antibody levels were shown to drop across all ages following

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[4] The term "more realistic" is intended to convey a higher SRR and therefore shorter estimates of the time to seroreversion. Bosomprah [27] showed an increase in the estimated SRR from 0.01393 (time to revert to seronegative approximately 72 years) to 0.0426 (23 years) using an extended catalytic model.

comprehensive interventions. Intuitively this drop in titer would occur before a drop in the proportion of seropositive individuals could be detected; thus, the resolution of antibody titre measurements allows for the detection of recent changes in malaria transmission.

In addition, crude antibody densities can be adjusted for age to determine whether an individual's response is higher or lower than the average for the population. This can be done by log-transforming antibody levels and fitting Loess curves to scatter plots of antibody response by age. Individuals are then assigned their residuals from linear regression, i.e., a measure of the extent of their difference from the average.[5]

Other Descriptive Measures for Malarial Serological Data

Further models have been utilized to examine patterns in serological data. Repeated measures on individuals allows for more realistic assessments of antibody boosting and decay events. Hidden Markov models (HMM) have been described in a longitudinal study, using a separate SRR for individuals who are seropositive at the start of a study (Bretscher et al. 2013). Furthermore, changes in responses over time can be investigated by looking at AUC or by categorizing individuals into categories of low, medium, and high responses (Cook et al. 2012; Wipasa et al. 2010).[6] This last method can be used to compare repeated cross-sectional surveys. Serological metrics used in other (vector-borne) infectious diseases – such as Chagas disease, dengue, and lymphatic filariasis – can be useful sources for additional descriptive statistics as well.

Applications of Serological Metrics

Here we discuss several applications of serological metrics which are widely discussed in the malaria literature and have proven to be useful at various levels of endemicity.

Assessment of Malaria Transmission Intensity
The SCR has been shown to correlate well with the EIR, clinical malaria incidence rates, and

malaria parasite carriage in several settings. Corran et al. showed considerable correlation between a range of EIR and SCR values in relation to altitude (Corran et al. 2007). Serological metrics and parasite carriage should be compared with caution, since antibody presence relates to recent or past exposure and parasite carriage relates to current exposure. One way to overcome this difficulty is by comparing the proportional changes in both the SCR and the prevalence of malaria infections for children aged 1–5 years old (representing recent transmission patterns) (Wong et al. 2014).

The SCR and crude seroprevalence measures have been used at various levels of endemicity and have proven to be less sensitive to seasonal changes or to fluctuations over the course of an infection in comparison with, e.g., the EIR and PR. They also show more granularity at low transmission levels, where these other metrics fail to discriminate. Intuitively, to accurately describe malaria transmission at lower transmission levels, serological metrics will require greater sample sizes in comparison with high transmission settings. However, in theory, these would still be smaller than sample sizes to accurately describe, e.g., parasite prevalence. Although sample size calculators have been proposed for malaria serological studies using seroprevalence or SCR (Sepúlveda et al. 2015), a formal comparison of sample sizes needed across different malaria metrics has not been performed to date.

Assessment of the Spatial Distribution of Malaria Exposure
The heterogeneity of malaria infections in a population is well established, with substantial differences in transmission levels between regions, villages, and even individual households. The importance of measuring this heterogeneity in transmission is widely recognized in the literature. The use of population-level malaria serological profiles in this context has been shown in various areas of endemicity and size. In relation to this, malaria transmission intensity is mapped spatially using serological data and has identified geographical foci of transmission in various endemic settings.

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[5] Furthermore, Reverse Cumulative Distribution Plots have been described as a methodology to analyse antibody data in 1995 [28] and applied in malaria epidemiological studies as a measure of change in transmission by Wong et al. [29]. More recently, mathematical models assessing the rate of antibody acquisition and loss using antibody density data have shown an increased precision of estimates of transmission patterns compared to

reversible catalytic models using dichotomous data [30–32].

[6] The reference Wipasa et al. is incorrect here as they do not discuss AUC, however this methodology was published recently by Arnold et al. [30]. A rectification has been sent to Springer.

Assessment of Risk Factors for Malaria Transmission

Apart from certain areas being more prone to malaria transmission – resulting in geographical foci – particular populations can also be at risk for malaria infection. In addition to age, exposure to mosquitoes due to work environment results in another population risk factor which is not as widely recognized in malaria research. However, there are a few studies which assessed the serological profile of an occupation-related malaria risk in mine workers and forest workers.

With more countries worldwide reaching a pre-elimination phase for malaria, the use of serology to screen immigrant populations can become increasingly important as well. In the absence of highly sensitive point-of-care parasite tests at present,[7] serological assays can be used to define occurrence of transmission in the past. This is especially useful when new antigens with different kinetic profiles are identified, which represent more recent exposure (antibody responses to antigens which decline within a known number of months after infection).[8]

The advantage of the presence of antibody responses over parasite presence to identify risk factors is the increased sensitivity owing to a prolonged evaluation period. The window to detect a current infection by parasite carriage is smaller and antibody responses can be evaluated years to decades later. At the same time, this also results in difficulties assessing historical versus current risk factors. Therefore, known risk factors for past exposure to malaria, especially age, should always be taken into account when analyzing new risk factors.

Assessment of the Effect of an Intervention

A reduction in transmission will result in lower exposure in children born since an intervention has been put into place. Over time, this will become evident in a population's serological profile. This will be more pronounced when control methods are implemented across a whole population and are universally effective, resulting in a sudden drop in exposure to infection. Whilst changes in transmission are often evident from seroconversion curves, the timing of the perceived

change does not always coincide with the time point of implementation. This is most likely due to differential rates of loss to antibodies between children and adults, such that children born and exposed to malaria before a reduction in transmission might not have mounted an effective memory immune response. The result is that the time of the effect of an intervention would be overestimated. Furthermore, when control methods are implemented more gradually or where coverage is less complete, the change in seroprevalence by age and in the SCR will also be more gradual. Thus a defined change point may be difficult to detect.

Assessment of Vector Contact

In addition to measuring serological evidence of exposure to the *Plasmodium* parasite, it is also possible to measure antibodies to exposure to mosquito bites (such as the mosquito saliva antigen gSG6). This may be useful as a measure of exposure of being bitten and thus to evaluate the effect of interventions intended to reduce mosquito exposure such as bed nets or IRS. Studies in Senegal found that vector control lowered levels of anti-gSG6 antibodies (Drame et al. 2013). Antibodies against the *Plasmodium* parasite and against mosquito antigen can be measured simultaneously which could be interesting in comparing the effect of different interventions. For example, both of these classes of antibodies should decline after vector control methods, but only antibodies against the parasite should decline following drug interventions.

Screening of Blood Donors

A range of commercial ELISA kits to test for malaria are available, which are most commonly used to screen blood donors (Chapter "▶ Blood Bank Screening – Bates") in non-endemic countries. In this setting, testing for serological evidence of past exposure to malaria is essential, especially considering the possible presence of dormant hypnozoites and the associated risks of donating blood while carrying these. Here again the difficulty of discriminating between a historical and current infection raises difficulties and markers of recent exposure would be advantageous.

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[7] A highly sensitive (hs) histidine-rich protein II (HRP2)-based RDT has recently been developed with a reported limit-of-detection that is 10-fold more sensitive than that of conventional RDTs (cRDT). Assessment of its

performance is ongoing in several field trials [33]. The only published results from a field trial to date are from eastern Myanmar where hs-RDT had a 2-fold greater sensitivity compared to cRDT while the specificity was similar using a combination of ultra-sensitive PCR and HRP2 ELISA as the reference standard. However, field sensitivity of the hs-RDT using the same combined reference standard was lower compared to laboratory assessments (35% versus 51%) which may be due to operational challenges [33,34].

[8] Helb et al. have described antibody responses that can predict recent exposure to infection in a cohort of Ugandan children in 2015 [35]. This work and other publications assessing antibody responses associated with recent infection will be discussed in the final section of the introduction (Section 5).

Future Directions

Epidemiological assessment of serological profiles can allow for detailed examination of malaria transmission dynamics in a population. The unique facet of creating a recent and historical overview of transmission by sampling a population across all ages is highly valuable in malaria research and for policy makers responsible for optimizing malaria control. The ability to detect a change point in transmission in a cross-sectional population is a useful way to measure the effect of interventions. In areas close to elimination serology has particular relevance as it is able to provide a greater granularity and range in comparison with parasite prevalence or EIR estimates. Moving forward in using serological measures for the evaluation of malaria transmission patterns in eliminating settings, definitions on how to accurately define "no transmission" need to be identified. Although at the moment only a population-level metric, future work should focus on individual patterns of antibody responses over time. Additionally, SRRs should be biologically confirmed in relation to age and transmission intensity in longitudinal field surveys in order to more accurately describe SCR.

Although testing the presence of antibodies using ELISA has high field applicability and low costs, it still typically involves an over 24 hour protocol due to incubation times. The use of lateral flow assays (LFAs), which – most commonly – test the presence of *P. falciparum* antigen expressed by infected red blood cells, requires minimal training, and results are ready within 15 minutes.[9] LFAs were originally designed to test symptomatic patients but are increasingly used in epidemiological studies. Ideally, these techniques would be combined to create a serology LFA that distinguishes between historical, recent and current infections which could be used in surveillance and screening. Commercial ELISA kits – at present used in the context of testing blood donors – have not been widely tested in the field. Experience using these tests is likely to increase with time, and their role will become clearer.

Serological measures can be used to identify populations or locations where interventions have been less effective and the targeting of these foci can help to reduce overall transmission further (Bousema and Drakeley 2011). Elliott et al. previously discussed research priorities for the development and implementation of serological tools for malaria surveillance (Elliott et al. 2014). They highlighted the need to identify novel and species-specific antigens and to develop standardized high-throughput assays.

In conclusion, serological tools have proven to be valuable adjunct measures of malaria transmission at all levels of endemicity. Serological data are increasingly collected as part of epidemiological field studies, and with DNA sequencing and protein microarray techniques identifying numerous novel antigenic targets, the role of immuno-epidemiology in malaria is likely to expand.

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[9] The LFA described here is what is referred to as RDT in other sections of this thesis. Hereafter, LFA will only be used for those detecting antibodies against the malaria parasite while RDT will only be used for those detecting malaria parasite antigens: *Plasmodium* lactate dehydrogenase (pLDH) or HRP2.

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4. Malaria Diagnostic Platform, Antibody Detection

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SECTION A – Student Details

Student	Lotus Leonie van den Hoogen
Principal Supervisor	Chris Drakeley
Thesis Title	The use of antimalarial antibodies to measure transmission in low transmission and pre-elimination settings

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Where was the work published?	Springer Link
When was the work published?	December 2015
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion	There were delays in the registration process which meant that the Sept 2015 deadline was missed. This chapter reviews antibody detection platforms and therefore formed as a basis of my introduction to their use and application in malaria research.

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Malaria Diagnostic Platform, Antibody Detection

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Synonyms

Serology; Immunoglobulin detection; Immunoassays

Definition

The measurement of antimalarial antibodies - to date - has had limited application in malaria diagnostics. However, it is a powerful tool for research purposes as it can detect both recent and historical transmission patterns at the population level. With the development of novel detection platforms, or immunoassays, the capacity to analyse antigenic targets has increased. This entry discusses the most commonly used antibody detection platforms in malaria research and overall assay practicalities.

Background

Antibodies, or immunoglobulins (Ig), are produced by plasma cells upon infection with a pathogen and are directed against specific antigenic targets. Secreted serum antibodies neutralize toxins, prevent the entry and spread of pathogens, and eliminate microbes. There are five Ig classes of which IgG has the highest serum concentration (Abbas et al. 2015) and has been most widely researched in the context of malaria. Antimalarial antibodies are produced during a first infection with the *Plasmodium* parasite and are boosted upon subsequent infections. Even under repeated exposure to malaria, sterile immunity is never complete, and it appears that continuous exposure to low-level parasitemia is required for protection to be maintained. It has been hypothesized that the antibodies secreted upon a first infection are produced by short-lived plasma cells, and reexposure causes long-lived plasma cells to produce a secondary antibody response that is faster, stronger, and of better quality (therefore mainly seen in adults) (Hviid et al. 2015).

In addition to mediating protection from disease, antimalarial antibodies can be used as a marker of exposure to malaria. Because antibody presence in an individual does not distinguish between a past or current infection, it is not a useful diagnostic to inform treatment or manage disease though there are potential applications in certain scenarios (see "Future Directions"). However, antibody detection is extremely useful in the context of malaria research, specifically immuno-epidemiology, and to identify possible risk from asymptomatic infections in blood donors (chapter
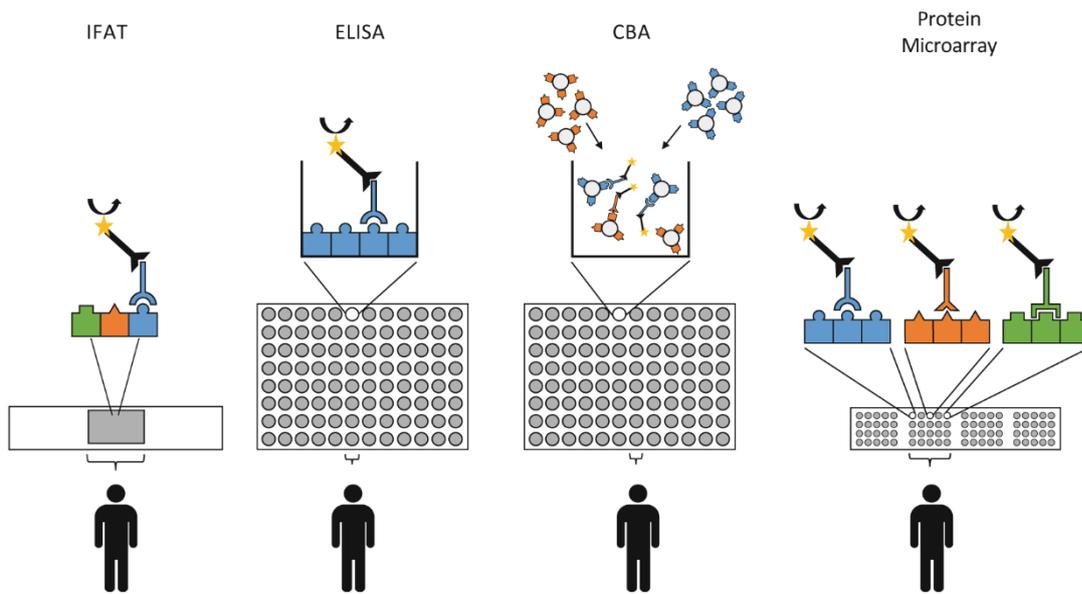
Malaria Diagnostic Platform, Antibody Detection, Fig. 1 Schematic of IFAT, ELISA, CBA, and protein microarray. *IFAT* immunofluorescence antibody test, *ELISA* enzyme-linked immunosorbent assay, *CBA* *cytometric bead array*. From *left* to *right* the number of identified antigenic targets – in relation to the number of individuals per test (i.e., per microscope slide or 96-well plate) – increases

"▶ Blood Bank Screening") (Warrell and Gilles 2002). While global trends of malaria transmission move to lower transmission and near eliminating, antibody detection methods may become increasingly important due to the ability to detect both recent and historical transmission patterns at the population level. Additionally, measuring antibody titers or short-lived antibody responses in a well-controlled assay will enable us to rapidly assess the effect of interventions. Details on the use of antibody responses in an epidemiological context are discussed in chapter "▶ Immuno-Epidemiology for the Evaluation of Malaria Transmission Patterns."

A variety of historical techniques to detect antibodies have largely been supplanted by enzyme-linked immunosorbent assay (ELISA), which has widespread use in malaria research due to its field applicability. More novel techniques to detect antibodies, including protein microarray and cytometric bead array (CBA),[10] have the advantage of being able to multiplex, i.e., test against multiple antigens simultaneously. Here, we focus on the most commonly

used antibody detection platforms in malaria research, immunofluorescence antibody test (IFAT), and ELISA and compare them to newer multiplex techniques. Furthermore, we discuss overall assay practicalities including sources of antibodies and antigens.

Measuring Antimalarial Antibodies

A detailed historical overview of serological assays used to measure antimalarial antibodies was presented by Drakeley and Cook (2009). This included methods such as complement fixation test, indirect hemagglutination assay, IFAT, ELISA, and protein microarray. Here, we expand on the most relevant of these for the current malaria research field – IFAT, ELISA, and protein microarray – and additionally discuss CBA. In general, all four serological assays discussed here are based on the same principal and can either detect antigen (direct or sandwich) or antibodies (indirect). An overview of the

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[10] Synonyms for the CBA in the literature are quantitative suspension array technology (qSAT), multiplex bead assay or array (MBA), and multiplex bead-based assay etc. Sometimes the brand name of the detection instrument is used: Luminex® or MAGPIX®. In this publication CBA was used, whereas in later thesis chapters MBA will be used.

Malaria Diagnostic Platform, Antibody Detection, Table 1 Test characteristics for IFAT, ELISA, CBA, and protein microarray. *IFAT* immunofluorescence antibody test, *ELISA* enzyme-linked immunosorbent assay, *CBA* cytometric bead array

Characteristic	IFAT	ELISA	CBA	Protein microarray
Dynamic range	+	+	+++	+++
Ability to multiplex	−	−	++	+++
Speed per sample/antigen	−/−	++/+	+/++	+++/+++
Amount of sample/antigen[a]	−/−	+/+	++/++	+++/+++
Costs[b]	−	+	++	+++
Field applicability	−	+++	+	−

[a]Amount of sample/antigen volume used; − = most sample/antigen used; +++ = least sample/antigen used
[b]Costs per identified antigenic target/sample; − = highest costs; +++ = lowest costs

assays discussed in this chapter is shown in Fig. 1 and Table 1.

Immunofluorescence Antibody Test (IFAT)

The IFAT procedure involves incubating human sera on a microscope slide on which whole parasitized red blood cells are fixed. A secondary antibody coupled with a fluorescent compound is added and the amount of fluorescence is examined using a florescence microscope. The major advantage of this method is the fact that it is relatively easy to perform. However, since fluorescence is determined by visual examination, it is difficult to standardize. Moreover, preparing separate slides per individual is labor intensive, and derived antibody responses are crude since whole parasite preparations are used (see below) (Drakeley and Cook 2009; Warrell and Gilles 2002).

Enzyme-Linked Immunosorbent Assay (ELISA)

In ELISA, antigens – for research these are typically single recombinant antigens whereas commercial kits (to screen blood products) use a pooled combination – are coated onto microtiter plates. Sera of interest are incubated in the plate after which a secondary (antihuman) antibody that is linked with an enzyme is added. In the final step, the enzymatic substrate is converted if there is bound enzyme present in the well. The resulting color change can be quantified by a spectrophotometer. Advantages of the ELISA protocol include high throughput capability,

field applicability, and low cost. Although the protocol is typically >24 h, a large number of plates can be run simultaneously and thus the time per sample is short. However, since antigens are tested individually and usually in duplicate or triplicate, the time per antigen tested is relatively long. Moreover, the dynamic range (i.e., the width from the minimum to the maximum value of the response signal) is narrow in comparison with the more novel techniques described below.

Cytometric Bead Array (CBA)

CBA is a relatively new technique which can measure the response to multiple antigens in a single sample simultaneously and is based on the principles of fluorescence-activated cell sorting (FACS). The assay uses beads with a specific spectral address, where each antigen is covalently linked to color-coded microspheres. Current models have the ability to perform up to 500 different tests in one sample. There are two lasers present in the unit, one identifies the bead region (or spectral address) and the other the magnitude of the derived signal which represents the proportion of bound antibodies. The ability to multiplex is one of the key strengths of the CBA, allowing the potential to test for multiple diseases at the same time. This could lead to highly efficient testing in the future; however, computational issues regarding the amount of data produced limit the use of both CBA and protein microarrays at present. Depending on machine settings, the time to read a single plate is relatively slow (1½–3 h), but since multiple antigens

are tested simultaneously, the relative time per antigen is fast. Furthermore, the dynamic range of CBA is wide which increases granularity for low antibody responses.

Protein Microarray

The protein microarray technique is similar to an indirect ELISA except 1000s of antigens may be screened simultaneously on one microarray slide and it has a greater dynamic detection range than ELISA. In the microarray, proteins of interest are placed (printed or dotted in nanogram to picogram amounts) onto specific glass slides, which are then coated to immobilize the protein. The slide is then exposed to a primary antibody (plasma or serum sample) and a secondary antibody (antihuman), with the last binding to a fluorescent label which can be detected using a microarray laser scanner (Schena 2005). The ability to detect a large range of antigens simultaneously makes the technique suitable for antigen discovery and broad assessments of the immune response to malaria. The time needed both per antigen and per plate is relatively fast, and – as in the CBA – small amounts of sera are needed. Limitations include correct quantification of the signal, potential signal variation between slides (e.g., due to variance in printed spot sizes or the chemical treatment of the slide), computational issues regarding the amount of data produced, and the fact that it is not field applicable.[11]

Assay Practicalities

Sources of Antibodies and Sample Collection Methods

The most frequently used source of antibodies in malaria serology research is serum which are increasingly collected as dried blood spots on filter paper from finger-prick blood sample (Corran et al. 2008). Although still an invasive technique, it is an attractive alternative to separating plasma or serum from a blood sample which may be impractical in remote field settings with limited laboratory facilities. Additionally, the same filter paper blood spots can be a source

of parasite DNA for PCR-based testing (Baidjoe et al. 2013). Blood spots are blotted onto filter paper which are then air-dried overnight at room temperature and stored with silica gel, preferably at $-20°$C. For serological analysis, discs with a known diameter are cut from the center of the bloodspot and eluted in buffer (Corran et al. 2008). Whatman 3 MM filter papers are the most robust and most frequently used in field surveys. Thinner papers (such as 1 MM) can also be used; however, multiple discs may need to be cut in order to ensure sufficient antibody concentration in the eluted sample. Plasma can also be spotted onto filter paper for ease of transport.

Less-invasive sources of antibodies are urine, tears, or saliva samples, of which saliva sampling has proven to be a potential alternative to measure malaria-specific antibodies (Estévez et al. 2011). Although saliva titers are generally lower than plasma titers and the assay needs to be standardized (e.g., sampling method and optimal saliva dilution), plasma and saliva malaria antibody levels have shown to be strongly correlated. Saliva antibodies may be particularly useful in repeated sampling studies. Tears have also been suggested as a possible source of IgG antibodies (Friedman 1990); however, its use for antimalarial antibody detection has not been formally investigated.

Finally, rapid diagnostic tests (RDTs) can be used as a source for antibodies and DNA and have proven to give comparable results for several malaria-specific antigens after storage at 4 °C (Williams et al. 2009). The use of RDTs for the recovery of antimalarial antibodies has economic and logistical advantages and can help generate rapid assessments of malaria transmission intensity alongside parasite prevalence measures.

Sources of Target Antigens

Crude Antigen Preparations
Historically, seroreactivity to malaria was assessed using whole parasites either fixed on microscope slides for IFAT or as crude protein preparations for Western blots or ELISA. *P. falciparum* schizont extract (*Pf*SE) is produced

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[11] I.e. comparatively high costs for equipment and consumables and the need for trained expert laboratory technicians.

using *P. falciparum*-infected red blood cells harvested from cultures of *P. falciparum* with parasites enriched at the schizont stage. There may be applications where crude antigen preparations represent a cheap and fast option; however, variability within laboratories and between endemic strains means this preparation is difficult to standardize. *Pf*SE has been used as an indicator of exposure to malaria infection in immuno-epidemiological malaria research (Cook et al. 2010; Ondigo et al. 2014). Although *Pf*SE preparations are highly immunogenic, seroreversion rates (i.e., the rate with which individuals become seronegative) have been shown to be high as well, as is to be expected since immunity against malaria is at most partially protective. Therefore, testing against a range of (recombinant) antigens would improve the sensitivity of malaria exposure estimates in comparison with whole parasite preparations (Ondigo et al. 2014).

Recombinant Antigens

Since the 1990s, recombinant culture using bacteria, yeast, baculovirus, or mammalian cell expression allows for the expression of specific target antigens. The discovery and elucidation of immunogenic, species-specific target antigens have huge potential for malaria research and diagnostic purposes. Much of the original focus was to identify possible vaccine targets though many of these targets have value as markers for assessing exposure to malaria. For example, the 19 k dalton merozoite surface protein 1 (MSP1$_{19}$) antigen, which had little effect when evaluated as a vaccine, has proven to be a valuable indicator of exposure to infection (Halbroth and Draper 2015). Most identified species-specific antigens are for *P. falciparum* and *P. vivax* – although cross-reactivity between these two is broad – while very few exist for *P. malariae*, *P. ovale*, and *P. knowlesi* (Elliott et al. 2014).

The identification of novel recombinant antigens is important for the malaria diagnostic field, but the process is complex. A novel target antigen can be identified through population genetics, genome sequencing, protein characteristics, and/or homology with other species. Alternatively, an

in vitro transcription and translation (IVTT) method can be used, which allows for high-throughput expression of targeted open reading frames. A disadvantage of this approach is that only ∼25 % of clones are sequence validated and conformational proteins may not be properly expressed (Doolan et al. 2008). While designing the protein construct, the structure and characteristics should be kept in mind (e.g., full length proteins are less stable and less soluble than truncated products or fragments). A protein construct is validated using DNA sequencing. Affinity chromatography is used to filter the purified antigen. It is also possible to filter by protein size or chemical features (e.g., charge); however, this option is less specific and more difficult to perform (Schena 2005).

Parasite Diversity and Antigenic Variation

Parasite diversity and antigenic variation result in immune evasion by the *Plasmodium* parasite (Warrell and Gilles 2002). Parasite diversity is caused by allelic polymorphisms in the genetic coding for antigens. These can lead to changes in critical epitopes and affect the specificity of antibody responses. *P. falciparum* merozoite surface protein 2 (PfMSP2), for example, has a number of allelic polymorphisms which, if expressed, are fixed throughout the course of an infection. In contrast, antigenic variation results in changes in antigens that can occur during an infection. Antigenic variation enables expression of different versions of a particular gene and thus ultimately results in changes in the expressed antigens or epitopes. For example, the var genes cause frequent switching in the expression of different *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) antigens which have a key role in cytoadherence and immune evasion (Singh et al. 2014). The equivalent for *P. vivax* are known as the vir genes.

Both immune evasion methods can be a potential problem while testing for the presence of malaria antibodies against a particular antigen. This may be overcome by including all known antigenic variants in the immunoassay or by making a chimera – fusing several epitopes of an antigen in one molecule – which has been done

for apical membrane antigen 1 (AMA1) (Remarque et al. 2008) and MSP1 block 2 (Tetteh and Conway 2011). Parasite diversity can also be useful: intuitively a change in transmission intensity will also cause a change in parasite diversity and thus measuring the number of variants of the parasite can be used as a measure of transmission patterns (Daniels et al. 2015).

Costs

Specific costs of assays will vary greatly based on costs for reagents, laboratory equipment, and region. Broadly speaking detecting antibody responses with ELISA is likely to be the cheapest assay if a small number of antigens is being tested and has the advantage of its field applicability. If more complex, multiplex, testing is done, equipment costs will be more expensive. However, multiplex approaches allow for the collection of large quantities of data simultaneously potentially leading to a more cost-effective assay. Moreover, in both CBA and protein microarray, very small amounts of antigens and sera are used which thus further reduces costs.

Other Assay Practicalities

Laboratory Factors

The main issue of antibody detection tests is standardization – interobserver as well as intraobserver variability can exist due to a variety of common laboratory factors (e.g., pipetting errors, temperature changes during testing, minor differences in the protocol such as incubation times or freeze-thawing of reagents). To minimize a number of these variations, some protocols advise a dilution series of positive control sample to generate a standard curve.[12] If this is included in every assay, this allows standardization between assays and between runs. Negative controls (e.g., serum from nonexposed donors) and blank samples should also be included to assess background reactivity and subtract this from test antibody responses.

The Positive Control

Most commonly a positive control with a known titer is used, within an appropriately controlled assay, to compare absolute antibody titers within longitudinal surveys and across studies. At present, antimalarial antibody titers are arbitrary in reflecting the level of antibodies in a sample. This is in part due to a lack of specific reagents as well as the complex nature of antibody reactivity in polyclonal sera where multiple epitopes are recognized with varying degrees of avidity.

Future Directions

Historically the development of antibody detection platforms has enabled us to test a larger number of samples against an increasing number of antigens simultaneously. The use of protein microarrays has led to the discovery of a wide range of novel malaria antigens. Moving forward, antibody responses against these novel antigens should be determined in epidemiological field studies in order to improve our knowledge of the human immune response to malaria. At present, the main issue of antibody detection is appropriate application of control samples in assays. The estimations and comparisons of antibody titers in a serum sample depend on the robustness (i.e., precision) of the assay used to detect antibodies. Standardized protocols should minimize this threat. Ideally, assays would include a positive control with a known titer in order to calculate absolute malaria antibody titers. This would enable us to compare antibody responses in longitudinal studies and across surveys more accurately.

Challenges for antibody detection lie in identifying *Plasmodium* species-specific immune responses. The overlap between *P. falciparum* and *P. vivax* sequences of AMA1 is more than 50 % and 45 % for $MSP1_{19}$ (Elliott et al. 2014). Future work should focus on identifying species-specific antigens, especially for *P. ovale*, *P. malariae*, and *P. knowlesi*,[13] in order to better distinguish between infections. Serological assays may have some use in specifically

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[12] Alternatively, a single dilution positive control sample or a single dilution of multiple positive control samples can be used.
[13] Herman et al. [36] and Krause et al. [37] have recently described *P. knowlesi*-specific proteins (both published in 2018).

identifying hypnozoite carriers for *P. vivax* and *P. ovale*.[14] In addition, instead of testing against single antigens, using a panel of multiple antigens would represent the human immune response more accurately. The choice of antigen also depends on transmission intensity, since highly immunogenic antigens may not be able to reflect recent changes in transmission at high endemicity (Elliott et al. 2014). Further epidemiological characterization of markers of serological incidence, i.e., target antigens associated with an infection that decline within a known number of months after infection (Helb et al. 2015), would be very advantageous. This would be specifically useful in the context of eliminating areas where all (recent) occurrences of transmission need to be identified.

Although antibody detection cannot be used as a point-of-care diagnostic, at the population level, it improves granularity as a measure of transmission in comparison with, e.g., parasite rates in low transmission and elimination settings. At present antibody profiles should be considered alongside other metrics that detect current infection and/or parasitemia. *Plasmodium*-specific antigenic targets with a range of kinetic profiles are needed in order to adequately describe transmission patterns at different levels of endemicity.

Cross-References

▶ Blood Bank Screening

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5. Recent Developments in Multiplex Antibody Detection and its Role in Improving Sero-Surveillance

The previous sections have summarised the limitations of existing metrics of malaria transmission in low endemicity settings. Submicroscopic infections (i.e. PCR positive, microscopy negative; defined here as low-density infections) are common in adults and at low transmission influencing the performance of PR measures. Whereas health-seeking behaviour, low-density and/or asymptomatic infections and the quality of the health system influence the sensitivity of passively collected data as a metric of ongoing transmission in communities. Antibody responses to well-characterised asexual stage antigens from malaria parasites represent cumulative exposure to infection and as such there is an increased sensitivity in detecting those exposed to malaria in cross-sectional surveys compared to repeated PR measures, which is especially of use at low transmission (**Figure 2**). Furthermore, the SCR has shown a stronger correlation with the EIR compared to PR measures (**Figure 3**).

Until recently, the majority of sero-surveillance data was based on a few well-characterised antigenic targets (using ELISA) whereby certain populations might have been missed due to the highly variable nature in which the parasite presents itself to its host and of human immunological responses to malaria. For example, sporozoite antigens elicit measurable antibody responses but their traversal from the skin to the blood stream and migration to the liver is rapid (i.e. 15 min to a few hours; reviewed in [39]). Antibody responses to this parasite stage may be more biased towards IgM or IgA and are likely lower in titre than IgG responses to antigens from replicating blood stages. Even within the blood compartment, responses to antigens appear to differ widely. Helb et al. showed that commonly used antibodies to antigens such as AMA-1 and MSP-1_{19} were not accurate predictors of recent infection or clinical incidence [35]. These targets are generally described as historical or long-term markers owing to the fact that antibody responses to these antigens have estimated half-lives of 23 years [27] to 50 years or more [40] for MSP-1_{19}, with limited data from observational studies suggesting 5-16 years (with 95% confidence intervals including infinity) [41]. As discussed, polymorphisms in the genetic coding for antigens is another example of the diversity in which the malaria parasite presents itself to its host which may affect antibody responses to specific antigens within and between resident populations exposed to different parasite populations. Age is a well-known human determinant of the acquisition and longevity of antimalarial antibody responses. Other examples of the variation in human immunological responses to malaria are the described differences in Fulani and Dogon ethnic groups in Mali. Fulani have shown higher IgM and IgG titres to a wider range of *P. falciparum* antigens while living in the same geographical area and under the same level of transmission as the Dogon [42]. In addition, Ubillos et al. recently described that, amongst other

factors, nutritional status and hemoglobin concentration significantly affected RTS,S/AS01E vaccine immunogenicity in infants and children from two sites in Ghana and Mozambique [43].

Multiplex antibody detection platforms have enabled us to test multiple antibody responses simultaneously in one sample. This may circumvent the problems described above by including a wider panel of antigenic targets to ensure that all those exposed to malaria are detected despite difference in parasite and human populations. It also allows for integrated sero-surveillance (i.e. to assess different pathogens [44]) as well as assessment of multiple malarial targets with different kinetics which increases the information from one cross-sectional survey [35,45].
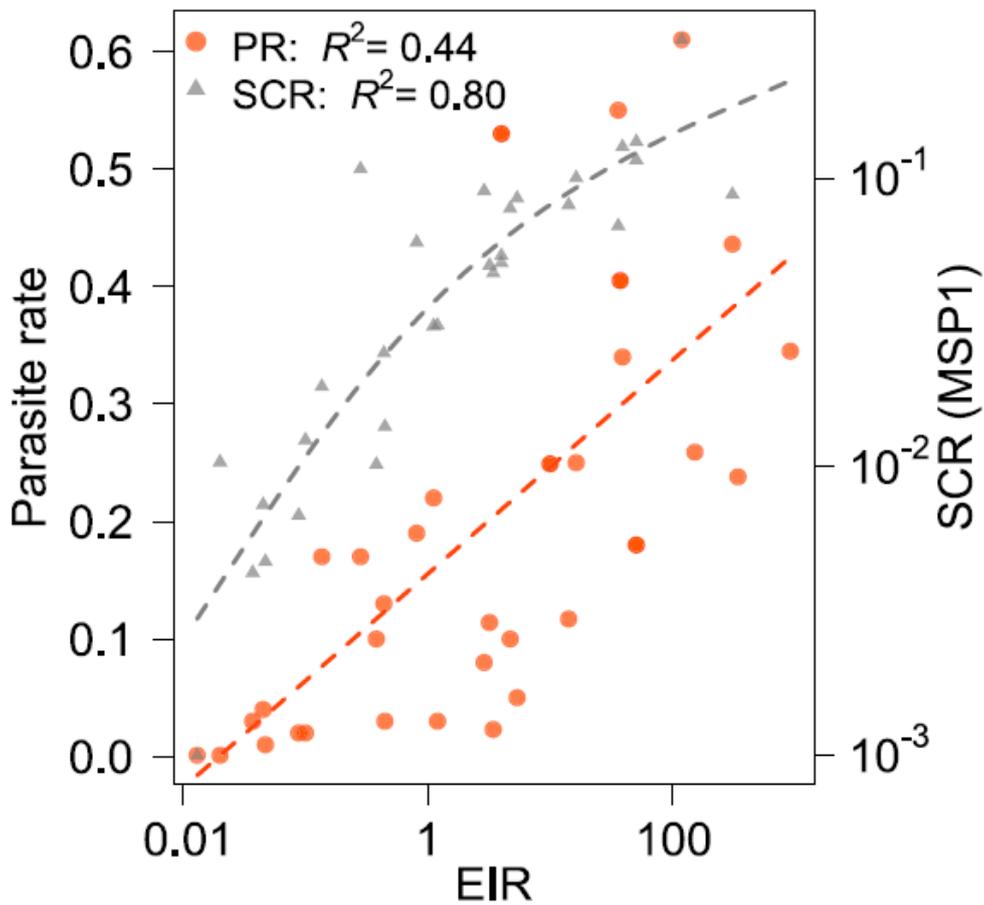
Figure 3: Comparison of EIR, SCR and parasite rate measurements from multiple African sites [46–56]. The seroconversion rate (SCR; using merozoite surface protein 1) has a stronger association with transmission (i.e. larger correlation coefficient; R^2), as measured by the entomological inoculation rate (EIR), compared to the parasite rate. Figure copied from Greenhouse et al. [45].

Outstanding issues

There are some outstanding issues for serological assays that need to be addressed which vary from technical (i.e. related to the assay) to analytical (i.e. related to the interpretation of results) and operational (i.e. related to the translation of results to operational actions); Figure 4 (adapted from Greenhouse et al. [45]). Some of these issues will be discussed below with an emphasis on serological metrics using multi-antigenic antibody responses but these do not cover all.

Firstly, the detection of multiple antibody responses simultaneously makes the assessment of assay variability between daily experiments and operators more difficult. The range of immunogenicity profiles of antibody responses mean that assay conditions will likely not be ideal for all. In addition, positive control hyper-immune sera are unlikely to show robust responses to all the antigens in the panel, depending on the number and type of antigens tested [57]. Secondly, to date, most serological metrics use antibody responses that are reduced to binary values which results in loss of information. The magnitude of the antibody response is an important factor in assessing a population's exposure history as under reduction of transmission, antibody levels will decrease along all ages but some individuals, especially adults, will not necessarily revert to a seronegative state (as repeated exposure has boosted antibody levels) [58,59]. Arnold et al. re-analysed the Garki project data [58], which was collected in an area of high malaria transmission and showed that antibody titres reduced in 0-20 year olds following IRS and mass drug administration (MDA), but seroprevalence only reduced among children <5 years old [30]. The use of continuous antibody metrics, however, calls for a well-standardised assay. Thirdly, (continuous or binary) antibody responses to up to 40 or 50 antigens have made it difficult to interpret results into actionable responses (e.g. do we intervene in a certain area? Is there remaining malaria transmission in this area? Has transmission changed in recent years?). As the use of multiplex antibody detection as a tool for epidemiological characterisation is relatively new, the interpretation and/or how to reduce information to manageable results is not yet validated. Although the hypothesis is that by using ELISA certain individuals who were exposed to malaria but did not show an antibody response to the one or two antigen(s) under investigation might have been missed, it is unlikely that we need as many as tens to fifty antigens to accurately describe malaria transmission patterns. Identifying a subset of antigens (i.e. 3-5) that combined can describe recent, intermediate and historical transmission would be advantageous.

The ultimate goal: use-case-scenarios for sero-surveillance

Greenhouse et al. (*under review*, Gates Open Research) have described five priority use-case-scenarios for antibody metrics in sero-surveillance: to document absence of transmission, for stratification of risk, to measure the impact of interventions, for decentralised immediate response, and for a *P. vivax* test and treat approach. For most of the use-case-scenarios, it is important that results are readily available and easy to interpret for quick turnaround (i.e. a clear framework for analyses). The optimisation framework of such an assay, from antigen availability to identification of the most informative combinations of responses, to validation of those responses in field trials, and finally, to the design of a point-of-care or lab-based antibody detection assay is shown in **Figure 4**. It should be noted that this framework (as well as the work discussed in this thesis) is dependent on variability in the production process, quality control, and ultimate availability of antigens which I will not assess in this thesis, however an overview of factors associated with antigen selection prior to analytical interpretation is shown in **Figure 5**.

Summary & thesis outline

The aim of this thesis is to assess the performance of antimalarial antibody metrics for active detection in low transmission and pre-elimination settings. Firstly, I will focus on other malaria metrics for active detection to identify where antibody metrics would be needed most. It has previously been shown that microscopy detects approximately 50% of all infections and these submicroscopic (or: low-density) infections are more common in adults and at low transmission [24,25]. To date, the performance of RDT relative to microscopy and PCR has not been compared in a comprehensive meta-analysis. Therefore, **Chapter 3** will aim to answer what the relationship is between PCR, RDT and microscopy prevalence estimates in asymptomatic populations using published and unpublished data from cross-sectional surveys in endemic settings.

Considering the extent of low-density infections at low transmission, adjunct metrics of malaria transmission should be investigated. Antibody responses represent past exposure to infection and, in combination with age, reflect a population's transmission history [40,55,60]. Antibody responses with relatively short half-lives may help to identify recent transmission patterns after a shorter period of time [35]. However, it is currently unknown if low-density infections induce measurable antibody responses, in particular to antigens associated with recent exposure. **Chapter 4** aims to answer which of forty, mainly blood-stage related, antigens induce measurable antibody responses using protein microarray and samples from participants who underwent low-density controlled human *P. falciparum* infections.

The development of multiplex antibody detection methods, such as protein microarray, has led to a vast amount of information across antibodies with different kinetic profiles. However, assay standardisation is challenging. Rogier et al. have recently described a multiplex bead assay (MBA) protocol which further improves throughput so that results from large-scale malaria surveys can directly inform control and elimination policies (Rogier et al., *in preparation*, **Appendix A**). In this protocol, sample (primary antibody) and anti-human secondary antibody are incubated simultaneously to reduce hands-on assay processing time and improve the ease-of-use of the assay. **Chapter 5** will aim to assess the precision of this recently described MBA protocol for antimalarial antibody detection, and will compare results to responses collected on a "conventional" MBA protocol (in which sample and anti-human secondary antibody are incubated consecutively with washes in between assay steps).

For certain use-case-scenario of antibody metrics (Figure 4), the design of a standardised, low-cost, high-throughput, easy-to-use assay for which results are readily interpretable, would be advantageous compared to interpreting results across multiple antibodies. In commercially available ELISAs, antigens are pooled to assess antibody responses for past exposure to malaria. These have been applied to screen blood products for evidence of malaria exposure prior to transfusion [61–65]. **Chapter 6** will aim to answer whether antibody measures from commercially available ELISA kits can be used to determine recent transmission patterns in an area of low transmission and pre-elimination.
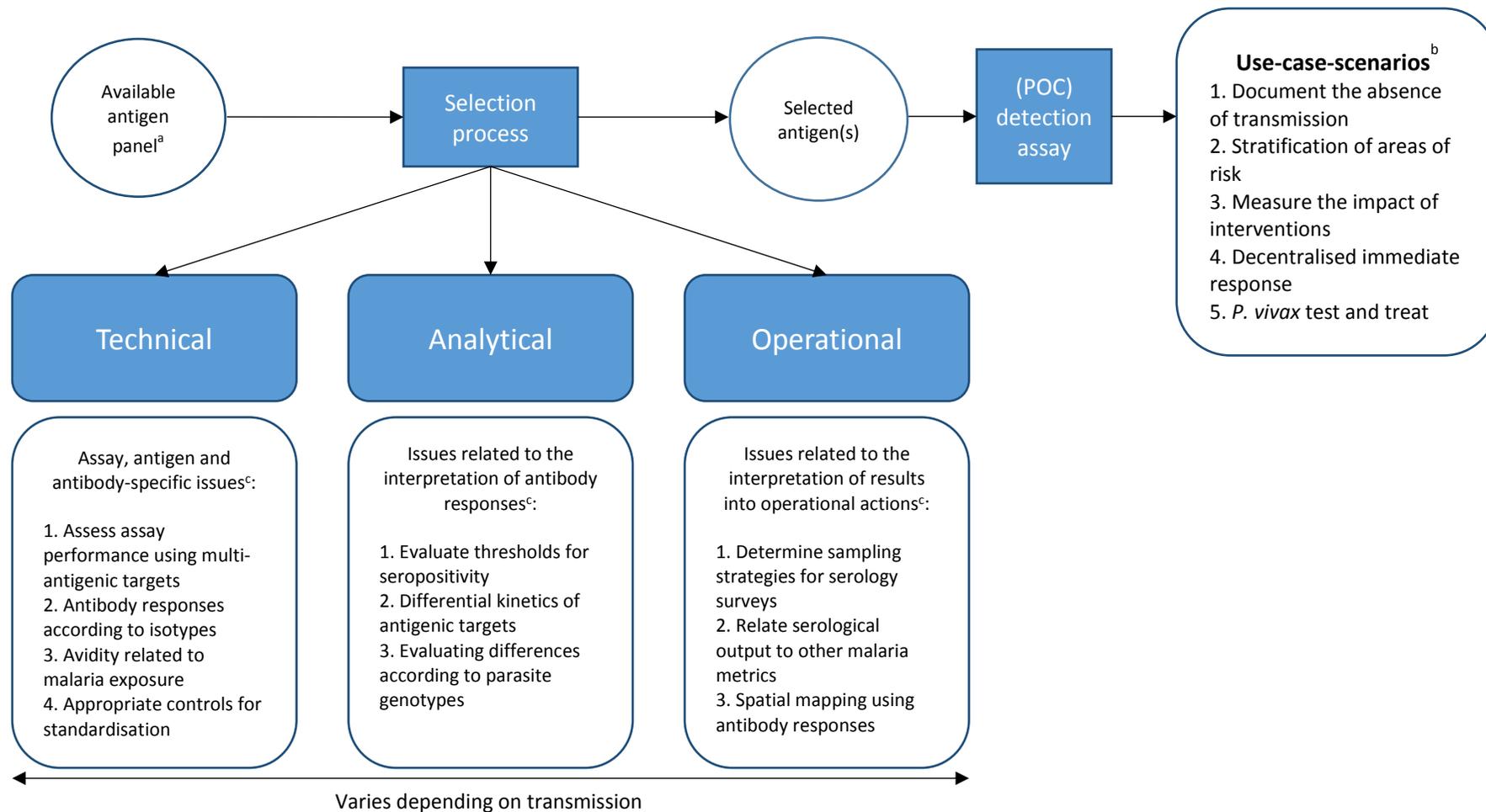
Figure 4: Schematic of optimisation framework of serological metrics and examples of outstanding issues. Adapted from Greenhouse et al. [45] [a] Factors associated with the available antigen panel prior to analytical interpretation are shown in Figure 7. [b] Use-case-scenarios were described by Greenhouse et al. (*under review*, Gates Open Research). [c] These are examples of outstanding issues, but it should be noted that this is not a complete list. POC: Point-of-care.

Antigen production
- Type of construct design: IVTT, population genetics, genome sequencing, protein characteristics (e.g. solubility) or homology with other species
- Expression system

Antigen fixation*
- Storage and stability of antigen
- Antigen concentration and batch-to-batch variation
- Selection of positive control pool to assess reactivity to antigen

Assay
- Variation between assay runs and operators (i.e. due to assay conditions, technician expertise, laboratory conditions, quality and stability of reagents as well as the selection of the positive control pool to assess this variation)

Figure 5: Flow-chart of factors that influence antigen selection prior to analytical interpretation. *To microtitre plate in ELISA, beads in multiplex bead assays or slides in protein microarrays.

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Chapter 2: Objectives

Objective

The research projects presented in this thesis had a general objective to assess the performance of antimalarial antibody metrics for active detection (i.e. cross-sectional populations) in low transmission and pre-elimination settings.

Rationale & specific objectives

Chapter 3

Microscopy detects about half of all malaria infections identified by PCR and these submicroscopic, low-density infections are more common in adults and at low transmission [1,2]. A recent analysis of Demographic and Health Surveys (DHS) data across Africa showed a higher prevalence of malaria measured by RDT compared to microscopy in 19 out of 22 surveys [3]. However, this study only included children under 5 years old and did not assess the effect of transmission intensity on discordance between these diagnostics. A large-scale, pooled analysis simultaneously comparing the concordance in prevalence estimates detected by microscopy, PCR and RDTs has not yet been performed.

A meta-analysis using PCR, RDT and microscopy prevalence estimates by geographical clusters sourced from literature review and unpublished cross-sectional data was conducted to determine the relationship between these estimates.

Specific objectives

- To examine the relationship between malaria prevalence measures obtained by PCR, RDT and microscopy for the detection of *P. falciparum* infections in endemic populations
  - o To determine the effect of transmission intensity on discordance in prevalence measures obtained by RDT compared to PCR
  - o To identify explanatory factors for discordance in prevalence estimates obtained by RDT compared to microscopy, and RDT compared to PCR, after adjusting for transmission intensity

Chapter 4

Antibody responses (IgG) have been suggested as an adjunct measure of malaria transmission as, in combination with age data, they can reflect both historical and recent transmission patterns [4–6]. They may prove particularly useful at low transmission as they represent cumulative exposure over time and thus are less sensitive to seasonal fluctuations in parasite rates and fluctuations in parasite density over the course of an infection. However, low-density infections are frequent at low transmission [1,2], and it remains unknown which antigens reliably induce measurable antibody responses to allow accurate detection of recent exposure to low-density infections. A powerful model to examine this is using controlled human malaria infections (CHMI) in which healthy volunteers are infected with the *Plasmodium* parasite.

Participant serum samples from eight previously performed CHMI studies were available one day pre-CHMI as well as one and two to seven months post-CHMI, and were tested for IgG responses using a protein microarray with forty antigenic targets, nearly all blood-stage related.

Specific objectives
- To identify antigens that induce measurable IgG responses following recent low-density *P. falciparum* infections in previously malaria-naïve CHMI participants
    o To determine if all participants induce measurable IgG responses to any of the forty antigenic targets assessed one month and two to seven months after challenge with malaria infection
    o To assess the number of antigenic targets needed to detect all participants with measurable antibody responses one month and two to seven months after challenge to malaria infection

Chapter 5

Measuring antibody responses to malaria can aid in describing malaria transmission patterns, especially at low transmission where infections and cases are infrequent. A pre-requisite to this is a standardised assay to compare antibody measures between surveys and populations. A suitable platform for this is multiplex bead assays (MBA) for antibody detection, which have recently been developed and applied to the malaria research field [7]. MBAs require a fraction of the reagent quantities, sample volumes and technician time compared to ELISAs to generate responses to many antigens [7,8]. Rogier et al. recently described the OneStep protocol in which sample and anti-human IgG are incubated simultaneously, which further increased the throughput of the MBA (Rogier et al.,

in preparation, **Appendix A**). The OneStep protocol showed similar sero-prevalence estimates to a Stepwise/conventional assay in which sample and anti-human IgG were incubated separately with washes in between assay steps. However, the precision of the assay and the comparison of continuous antibody measures between protocols has not yet been evaluated.

Chapter 5 uses data collected in three large-scale malaria transmission surveys in Haiti. A standard of Haitian hyperimmune sera was pooled and a 6-point dilution series was included on each plate alongside participant samples. Furthermore, a 6-point dilution series of the *P. falciparum* WHO reference standard 10/198 [9] was included on one plate per day. These standard curves were used to assess assay precision. Lastly, 804 participant samples were tested on both the OneStep and Stepwise MBA protocol to determine the relationship in antibody measurements between these protocols.

Specific objectives
- To assess the applicability of the high-throughput OneStep MBA protocol for IgG data collection for large-scale malaria transmission surveys
    o To determine if the OneStep MBA protocol can be used as a high-throughput tool in collecting IgG data for participant samples
    o To assess the precision of the OneStep protocol as determined by repeated measures from standard curves of positive control hyperimmune sera included on each plate
    o To determine the relationship between continuous measurements of antibody responses between the OneStep and a Stepwise/conventional MBA protocol

Chapter 6

As previously mentioned, a standardised assay is essential to compare serological results across studies and populations. However, there are no standardised assays to measure malaria antibodies for epidemiological use. There are several commercially available ELISA assays in which antigens are pooled, but these have been developed to screen blood donations for evidence of malaria exposure prior to transfusion [10–15]. Some have applied these in an epidemiological context, such as in Ethiopia [16]. However, to our knowledge, a comparison of the performance of multiple commercially available ELISAs for epidemiological characterisation of malaria transmission has not yet been carried out.

Chapter 6 will firstly discuss the applicability of commercially available ELISA kits for epidemiological characterisation of transmission. This consisted of the costs per sample, the amount of serum needed

to test a sample, specificity using serum samples from malaria unexposed individuals, and cross-reactivity using serum samples from *Toxoplasma*-infected individuals. Furthermore, a composite measure of ease-of-use was created based on total incubation time, reagent preparation and other practical considerations. Finally, the performance of antimalarial antibody measurements using commercially available ELISA kits in describing transmission was assessed by testing samples from Praia, Cape Verde (low transmission) and Bataan, the Philippines (pre-elimination).

Specific objectives

- To determine if antimalarial antibody measures from commercially available ELISA kits can be used to describe transmission patterns in a low transmission and pre-elimination area
    - o To assess the cost/sample, amount of serum needed per sample, specificity, cross-reactivity to *Toxoplasma,* and ease-of-use of commercially available ELISA kits for antimalarial antibody detection
    - o To compare antimalarial antibody measures from commercially available ELISA kits with (historical) malaria case counts from health facilities in a low transmission and pre-elimination area
    - o To assess the costs/sample, amount of serum needed per sample, specificity, cross-reactivity to *Toxoplasma* and ease-of-use of an established research-based ELISA for antimalarial antibody detection
    - o To compare antimalarial antibody measures from an established research-based ELISA with (historical) malaria case counts from health facilities in a low transmission and pre-elimination area

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Chapter 3: Comparison of Diagnostics for the Detection of Asymptomatic *Plasmodium falciparum* Infections to Inform Control and Elimination Strategies

LONDON SCHOOL of HYGIENE &TROPICAL MEDICINE

RESEARCH PAPER COVER SHEET

PLEASE NOTE THAT A COVER SHEET MUST BE COMPLETED FOR EACH RESEARCH PAPER INCLUDED IN A THESIS.

SECTION A – Student Details

Student	Lotus Leonie van den Hoogen
Principal Supervisor	Chris Drakeley
Thesis Title	The use of antimalarial antibodies to measure transmission in low transmission and pre-elimination settings

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?	Nature		
When was the work published?	December 2015		
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion	There were delays in the registration process which meant that the Sept 2015 deadline was missed. This work is included as it was an important part of my understanding of the use of different malaria metrics. The knowledge I gained of different malaria research groups and their projects through the literature review, and it also led to me being part of the MalERA Refresh process as rapporteur.		
Have you retained the copyright for the work?*	Yes, CC-BY	Was the work subject to academic peer review?	Yes

*If yes, please attach evidence of retention. If no, or if the work is being included in its published format, please attach evidence of permission from the copyright holder (publisher or other author) to include this work.

SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	
Please list the paper's authors in the intended authorship order:	
Stage of publication	Choose an item.

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	Co-first-author with Lindsey Wu. Both authors contributed equally to analyses and writing.

Improving health worldwide

Student Signature: ▆▆▆▆▆▆▆▆▆▆ **Date:** 28/11/2019
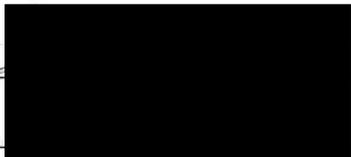
Supervisor Signature: ▆▆▆▆▆▆▆▆▆▆ **Date:** 25/11/2019

ARTICLE OPEN

Comparison of diagnostics for the detection of asymptomatic *Plasmodium falciparum* infections to inform control and elimination strategies

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Chris J. Drakeley[1] & Lucy C. Okell[2]

The global burden of malaria has been substantially reduced over the past two decades. Future efforts to reduce malaria further will require moving beyond the treatment of clinical infections to targeting malaria transmission more broadly in the community. As such, the accurate identification of asymptomatic human infections, which can sustain a large proportion of transmission, is becoming a vital component of control and elimination programmes. We determined the relationship across common diagnostics used to measure malaria prevalence — polymerase chain reaction (PCR), rapid diagnostic test and microscopy — for the detection of *Plasmodium falciparum* infections in endemic populations based on a pooled analysis of cross-sectional data. We included data from more than 170,000 individuals comparing the detection by rapid diagnostic test and microscopy, and 30,000 for detection by rapid diagnostic test and PCR. The analysis showed that, on average, rapid diagnostic tests detected 41% (95% confidence interval = 26–66%) of PCR-positive infections. Data for the comparison of rapid diagnostic test to PCR detection at high transmission intensity and in adults were sparse. Prevalence measured by rapid diagnostic test and microscopy was comparable, although rapid diagnostic test detected slightly more infections than microscopy. On average, microscopy captured 87% (95% confidence interval = 74–102%) of rapid diagnostic test-positive infections. The extent to which higher rapid diagnostic test detection reflects increased sensitivity, lack of specificity or both, is unclear. Once the contribution of asymptomatic individuals to the infectious reservoir is better defined, future analyses should ideally establish optimal detection limits of new diagnostics for use in control and elimination strategies.

Over the past two decades, considerable progress has been made in reducing the global malaria burden. Between 2000 and 2013 alone, malaria-related mortality decreased by 47% worldwide and 54% in Africa. In addition, more than half of malaria endemic countries are on track to meet global targets to reduce malaria incidence by 75% in 2015 (ref. 1). These achievements are largely due to the widespread use of insecticide-treated nets (ITNs) and highly effective antimalarial treatments. The treatment of symptomatic cases in particular has been enabled by notable advances in the development and deployment of more accurate malaria diagnostics[2,3]. However, efforts to reduce the burden of malaria infections further in the future will require moving beyond the treatment of clinical infections to targeting transmission more broadly in the community. As such, the accurate identification of asymptomatic human infections, which can sustain a large proportion of transmission, is becoming a vital component of control and elimination programmes[2,4].

Community chemotherapy (for example, mass screen and treat (MSAT) or mass drug administration (MDA) programmes) in conjunction with ongoing vector control is an approach under consideration for the interruption of transmission. This is achieved through the direct treatment of potentially infectious individuals. In the case of MSAT strategies, delivering drugs specifically on the basis of positive test results may be considered preferable to presumptive treatment because it provides clear benefit to the recipient and limits excess drug use that may drive antimalarial resistance. However, owing to the insufficient sensitivity of existing field diagnostics used to identify asymptomatic infections, studies have shown that MSAT has limited effect in reducing transmission[5,6].

Measuring parasite infection by microscopy has been the gold standard in malaria research for more than a century and remains relatively widespread as a point-of-care diagnostic in clinical and epidemiological settings. More recently, the advent of rapid diagnostic tests (RDTs), which measure the presence of histidine-rich protein 2 (HRP2) for *Plasmodium falciparum* and/or lactate dehydrogenase for other *Plasmodium* species (pLDH), has expanded the range of diagnostic options. Originally developed to inform clinical treatment, RDTs are increasingly important for epidemiological characterization[7] because of their low cost and field applicability. However, most only have reported detection limits in the range of 100 to 200 parasites per microlitre[8,9] in comparison with around 50 parasites per microlitre by expert microscopy[10].

Over the past three decades, the development of nucleic acid amplification tests has improved the detection limit for malaria infection to less than 1 parasite per microlitre by ultrasensitive quantitative polymerase chain reaction (qPCR)[11,12]. Although these detection thresholds are more appropriate for
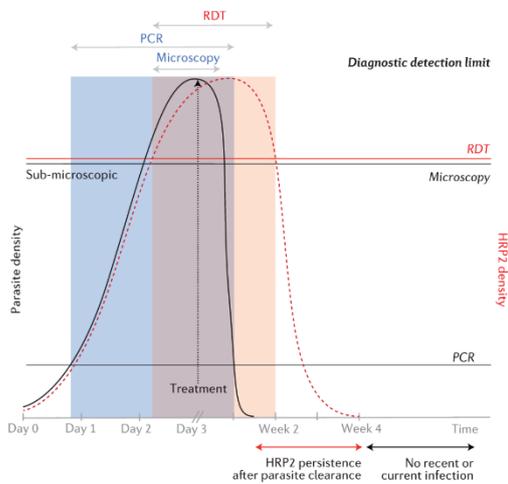
Figure 1 | Schematic of diagnostic detection limits with respect to parasite and HRP2 density. The black curve indicates parasite density and the red curve indicates HRP2 density. Time scale is in days prior to treatment and in weeks after treatment. Horizontal lines are the detection limits of respective diagnostics. The blue shaded area shows detectability of parasites by microscopy and/or polymerase chain reaction (PCR), whereas the red shaded area shows detectability of HRP2 by rapid diagnostic test (RDT).

measuring low-density infections than microscopy and RDTs, most PCR techniques remain impractical for wide-scale use in field surveys owing to cost, processing time and the lack of appropriate laboratory facilities in many endemic countries[10]. Comparative analysis of malaria prevalence, measured by both microscopy and PCR in cross-sectional surveys, has shown that sub-microscopic low-density infections are common across a range of transmission settings[13,14]. These infections may be chronic and asymptomatic, particularly in previously exposed individuals with more mature immune responses. More importantly, even at low parasite densities, they are still capable of infecting mosquitoes and seeding onward transmission[15]. Even though RDTs are becoming more common in areas where these types of infections are prevalent, studies formally evaluating their performance in detecting asymptomatic infections remain scarce.

Recently, there has been an increased focus on developing improved diagnostics to inform malaria elimination strategies. The analysis presented in this paper aims to determine the concordance of current malaria diagnostic methods, forming a baseline to evaluate further how they can be improved to inform malaria control and elimination strategies. It should be noted that, in principle, quantifying the presence of gametocytes is considered the most accurate method for characterizing transmission and the potential infectiousness of individuals. Research in this area is ongoing, but the technical challenges of existing gametocyte assays preclude them from standardized use[16]. Moreover, all malaria infections have the capacity to produce gametocytes[17,18]. Therefore, in the context of community chemotherapy programmes, any individual who tests positive for asexual parasites should be treated to reduce transmission. Given this operational framework, this paper does not address the role of diagnostics that specifically measure gametocytaemia.

So far, no studies have comprehensively evaluated the concordance across PCR, RDT and microscopy detection methods simultaneously in asymptomatic populations. Although microscopy- and PCR-measured prevalences are based on similar biological endpoints (parasite density), diagnostic results based on RDTs are less comparable given that HRP2 and pLDH are indirect measures of parasite biomass[19]. HRP2 can persist in the blood for up to two weeks after parasite clearance[20]. Consequently, results across these diagnostic methods indicate a range of possible infection states, from patent or sub-microscopic

infection to recently cleared infection (Fig. 1). A limited number of studies have reviewed the detection capability of RDTs in asymptomatic individuals[8,21], but key research questions still remain. A recent analysis of Demographic and Health Surveys (DHS) across Africa showed a higher prevalence of malaria when measured by RDTs compared with detection by microscopy in 19 out of 22 surveys. This report also highlighted the issue of false positives owing to prolonged presence of HRP2 after parasite clearance[21]. However, studies have not reviewed the detection capability across all three diagnostics. Furthermore, the DHS study only considered children under 5 years of age and did not determine the effect of malaria transmission intensity on diagnostic discordance. This is particularly important given that low-density infections seem to be most common in adults and in low-transmission settings[13,14].

In this study, we determine the relationship across malaria prevalence measures obtained by current diagnostic methods — PCR, RDT and microscopy — for the detection of *P. falciparum* infections in endemic populations based on a pooled analysis of published and unpublished cross-sectional data.

METHODS

Literature review and data collection. We carried out two separate literature reviews to identify studies in which *P. falciparum* prevalence was measured by different diagnostic techniques in the same individuals: first, by RDT and microscopy, and, second, by RDT and PCR. Relevant studies were identified in PubMed and Embase, using MeSH and Map terms when possible. For the RDT and microscopy review, the search terms were: "'rapid diagnostic test' and 'microscopy' [MeSH/Map] and 'malaria falciparum' [MeSH/Map]", and for the RDT and PCR review the search terms were: "'polymerase chain reaction' [MeSH/Map] and 'malaria falciparum' [MeSH/Map]". Searches were limited to English, human and post-2005 (considering the substantial development in RDTs over time[22]). For Embase, the searches were also limited to journal articles. Inclusion criteria were applied as previously described[13]. In short, only studies that were cross-sectional (on populations not selected according to malaria test results or symptoms), that were of populations from a malaria endemic region, that used RDTs targeting *P. falciparum* only or mixed infections (HRP2 and/or pLDH) and that used PCR or loop-mediated isothermal amplification (LAMP) methods were included. For intervention studies, only baseline data were included, except for treatment studies where a sufficient amount of time had passed between last treatment and follow-up. Separate publications that used the same data set or measured 0% prevalence by both methods were removed, as well as data from clusters with fewer than five individuals. RDT and microscopy studies identified in our literature search that also included PCR measurements were included in the RDT and PCR data set, and vice versa for RDT and PCR studies that included microscopy measurements. In addition to the literature review, we sought as many individual-level data sets as possible from studies with the above inclusion criteria.

RDT and microscopy. Where available, information on location, sample size, RDT brand and type (HRP2 or pLDH), age group (15 or younger compared with older than 15) and prevalence estimates were recorded[5,23–42]. Furthermore, data from the DHS online database were extracted[43]. These included individual-level data on location and timing of collection, RDT and microscopy test results, RDT brand[21], age, sex, use of an ITN, fever and antimalarial use in the past two weeks. In addition, individual-level data sets from one unpublished and one published study were included[44], as well as shared data sets of the RDT and PCR comparison that also included microscopy measurements (see below)[45–49].

RDT and PCR. Corresponding authors of the 13 studies identified from the literature search were contacted to request individual-level data in December 2014 and reminders were sent out 4 weeks later. Of the contacted authors, six responded within the timeframe; five data sets were included[45–47,49,50], and one data set had been destroyed for privacy compliance. Prevalence measures and study information (including PCR method) were extracted as described above from the publications in the aforementioned literature search and the non-responders group, as well as included studies from the RDT and microscopy search that also reported PCR proportions[25,27,34,39,40,42,51–55]. Four additional individual-level unpublished and published data sets were included[44,48].

Statistical analyses. We analysed the association between PCR- and RDT-measured prevalence, and microscopy- and RDT-measured prevalence by fitting a linear relationship on the log odds scale[13,56]. Prevalence (on a scale of 0 to 1) was defined as $\frac{e^{(\log odds)}}{1+e^{(\log odds)}}$, where $\log odds = \log_e\left(\frac{prevalence}{1-prevalence}\right)$

$$\Omega_{Ri} = \Omega_{Pi} + \delta_{Ri} \qquad (1)$$

$$\delta_{Ri} = \delta'_{Ri} + \beta_0 \left(\Omega_{Pi} - \bar{\Omega}_P\right) \qquad (2)$$

$$\Omega_{Ri} = \Omega_{Mi} + \delta_{Ri} \qquad (3)$$

$$\delta_{Ri} = \delta'_{Ri} + \beta_0 \left(\Omega_{Mi} - \bar{\Omega}_M\right) \qquad (4)$$

In Equations 1–4, Ω_{Ri} is the log odds of RDT-measured prevalence in trial i, Ω_{Pi} is the log odds of PCR prevalence, Ω_{Mi} is the log odds of microscopy-measured prevalence, δ_{Ri} is the log odds ratio (OR) of RDT- to PCR-measured prevalence (RDT:PCR; Equation 1) or RDT- to microscopy-measured prevalence (RDT:microscopy; Equation 3), δ'_{Ri} is the expected log OR of RDT:PCR prevalence (Equation 2) or RDT:microscopy prevalence (Equation 4) when the log odds of PCR- or microscopy-measured prevalence is equal to the mean across trials, $\bar{\Omega}_P$ and $\bar{\Omega}_M$ are the mean log odds of PCR- and microscopy-measured prevalence, respectively, across trials, and β_0 is the regression coefficient. To allow for varying sample size and sampling variation across the surveys included in our analysis, the model was fitted using Bayesian Markov Chain Monte Carlo methods in JAGS version 3.4.0 and the *rjags* package in R version 3.0.2 (ref. 13). We also explored fitting polynomial relationships, but these provided no substantial improvement in fit to the data over the linear model as assessed by deviance information criterion, nor were these fitted relationships qualitatively different (data not shown). To confirm that the fitted curves at different prevalence ranges were not overly influenced by the high number of data points in lower transmission areas, we fitted separate relationships in three PCR-measured prevalence bands: <5%, 5–20% and >20%. These categories represent approximate cut-offs that have been suggested as thresholds for operational decision-making. Broadly speaking, programmes can begin to consider targeted and focal control strategies when parasite prevalence by microscopy falls below 5% (ref. 57), which translates to a PCR-measured prevalence of 20% (ref. 14), and move towards targeted elimination when it falls below 1% (ref. 58) (5% PCR-measured prevalence[14]).

We also conducted a meta-analysis of the risk ratio between RDT:PCR prevalence or RDT:microscopy prevalence, adjusted for random effects at the study level (for RDT:PCR) or country level (for RDT:microscopy). Studies that reported zero infections by either diagnostic method were assigned a value of 0.01 to allow a risk ratio to be calculated. To evaluate the effect of explanatory factors on discordant test results, individual-level data were analysed by logistic regression, allowing for random effects at the study or country level as noted above. The meta-analysis was done with the *metafor* package in R version 3.0.2, and the logistic regression with the *logit* command in STATA version 13.

We assessed the ability of our models to predict RDT-measured prevalence based on microscopy- or PCR-measured prevalence data. Leave-one-out cross validation was used to evaluate the RDT:PCR and the RDT:microscopy models separately. The data available for direct comparison of malaria detection by RDT and PCR in the same individuals were sparse relative to the quantity of data available for the RDT:microscopy and previous microscopy:PCR comparisons. Therefore, we also triangulated the relationship between RDT- and PCR-measured prevalence by combining the RDT:microscopy relationship calculated in this study with the microscopy:PCR prevalence relationship that has been previously defined[13]. The credible interval of the triangulation line was computed from the posterior distributions of all the parameters from both equations combined. We evaluated whether this triangulated RDT:PCR relationship was significantly different from the observed RDT:PCR relationship using the posterior distributions of the predictions from each model.

RESULTS

Literature search and data collection. The literature search generated 549 results in Pubmed and an additional 37 in Embase for RDT and microscopy, and 2,247 results in PubMed and an additional 426 in Embase for RDT and PCR. In
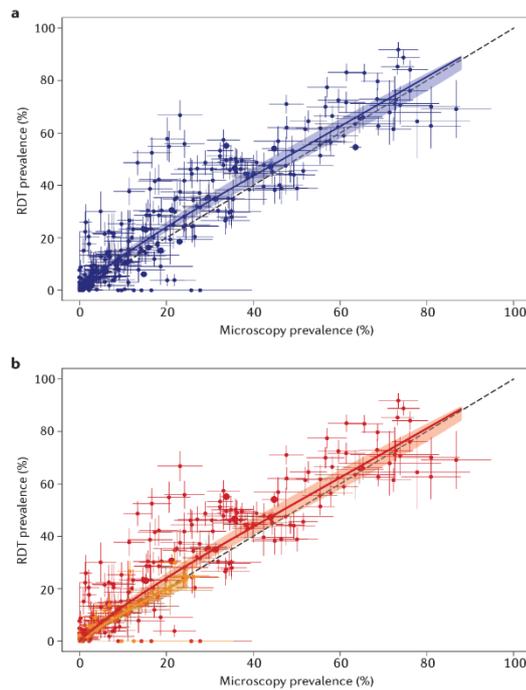
Figure 2 | The relationship between rapid diagnostic tests (RDTs) and microscopy *Plasmodium falciparum* prevalence overall (**a**) and stratified by age group (**b**). In **b** red indicates children (those under 15 years) and yellow indicates adults (those over 15 years). Dashed lines indicate the expected relationship if RDT and microscopy detected equal prevalence. Horizontal and vertical lines indicate 95% confidence intervals around point estimates, whereas coloured solid lines indicate the median of the Bayesian posterior distributions from the fitted model and shaded areas indicate 95% credible intervals. Radius of point estimates indicate cluster size (from small to large: <100, 100–1,000 and >1,000).

total, 20 RDT: microscopy studies and 13 RDT:PCR studies from the literature search met our inclusion criteria. Combined with additional data sets from DHS and unpublished studies, the pooled data available for evaluation yielded 323 pairs of prevalence estimates for RDT and microscopy[5,23–42,44–49] and 162 pairs for RDT and PCR[25,27,34,39,40,42,45–55]. The extracted proportions together with the main characteristics of the studies from our literature search are provided in the Supplementary Information. The main PCR method used was nested PCR (nPCR; 15 of 20) of which mainly the Snounou method[59] was used (11 of 15). The other methods included LAMP (1 of 20) and qPCR (4 of 20). All of the included RDTs in both comparisons were based on HRP2, with 8 out of 20 studies also including pLDH to measure species other than *P. falciparum*. However, this study only focuses on the detection of *P. falciparum* infections.

Comparison of RDT- and microscopy-measured prevalence. Analysis of RDT- and microscopy-measured prevalence included data from 172,281 individuals who were tested with RDTs (cluster prevalence range = 0–92%) and 186,434 tested with microscopy (cluster prevalence range = 0–87%). The 323 geographical clusters spanned a total of 29 countries (cluster size range = 5–7,664). Overall, prevalence of *P. falciparum* measured by microscopy detected 87% (95% confidence interval (CI) = 74–102%) of RDT-positive infections. Therefore, RDT and microscopy detection was comparable (Fig. 2a, Table 1), with less of a difference between the two diagnostic methods in children under 15 years of age (77%, 95% CI = 71–85%) compared with adults (over 15 years)

Table 1 | Best fit relationships between RDT:microscopy and RDT:PCR prevalence.

RDT:microscopy
OVERALL
log odds RDT prevalence = 0.108 + 0.907 × log odds microscopy prevalence (all ages)
BY AGE CATEGORY
log odds RDT prevalence = 0.109 + 0.908 × log odds microscopy prevalence (under 15 years)
log odds RDT prevalence = −0.168 + 0.890 × log odds microscopy prevalence (over 15 years)
RDT:PCR
OVERALL
log odds RDT prevalence = −0.968 + 1.186 × log odds PCR prevalence (all ages)
BY AGE CATEGORY
log odds RDT prevalence = −0.382 + 1.306 × log odds PCR prevalence (under 5 years)
log odds RDT prevalence = −0.864 + 1.213 × log odds PCR prevalence (6–15 years)
log odds RDT prevalence = −1.378 + 1.300 × log odds PCR prevalence (over 15 years)
BY AGE CATEGORY
log odds RDT prevalence = 1.097 + 1.690 × log odds PCR prevalence (<5% prevalence)
log odds RDT prevalence = 0.211 + 1.754 × log odds PCR prevalence (5–20% prevalence)
log odds RDT prevalence = −0.516 + 1.904 × log odds PCR prevalence (>20% prevalence)
PCR PREVALENCE BASED ON DATA TRIANGULATION
log odds PCR prevalence = 0.108 + 0.907 × [(log odds RDT prevalence − 0.954)/0.868]

PCR, polymerase chain reaction; RDT, rapid diagnostic test.

(60%, 95% CI = 48–86%) (Fig. 2b, Table 1). The lower age-specific risk ratios are due to smaller cluster sizes after stratifying the data by age group. However, regression analysis of individual-level data did not show a significant association between age group and test discordance (Supplementary Table 1).

Effect of individual level covariates on RDT:microscopy discordance. In addition to age, we explored the effect of several other covariates on diagnostic outcomes, and adjusted for transmission intensity as assessed by microscopy-measured prevalence (Supplementary Table 1). A significant association was seen between self-reported antimalarial use in the two weeks before survey testing and RDT positivity in individuals who tested negative by microscopy (OR = 1.71, 95% CI = 1.16–2.51, *p* = 0.006). The presence of fever at the time of testing (recorded temperature with study-specific cut-off or self-reported) reduced the odds of undetected malaria infection by RDT among microscopy-positive individuals (OR = 0.59, 95% CI = 0.39–0.89, *p*<0.001). Among individuals testing negative by microscopy, presence of a fever was significantly associated with RDT positivity (OR = 1.84, 95% CI = 1.51–2.24, *p*<0.001), after adjusting for transmission intensity. There was a borderline significant increased risk of malaria infection being undetectable by RDT among those who used an ITN and were microscopy positive (OR = 1.26, 95% CI = 1.00–1.59, *p* = 0.053), whereas use of an ITN was associated with decreased RDT positivity (OR = 0.84, 95% CI = 0.73–0.97, *p* = 0.019) among microscopy-negative individuals. There was no evidence of an association between RDT brand and the risk of an undetected malaria infection by RDT among microscopy-positive individuals. Among microscopy-negative individuals, the proportion testing positive was different between RDT brands, but these results are difficult to interpret, owing to complete correlation between study and RDT brand. The year of the survey was not associated with discordant test results for RDT:microscopy.

Comparison of RDT- and PCR-measured prevalence. Analysis of RDT- and PCR-measured prevalence included 35,887 individuals tested with an RDT (cluster prevalence range = 0–45%) and 31,178 individuals tested with PCR (cluster prevalence range = 0–52%). There were a total of 162 geographical clusters across 17 countries (cluster size range = 5–3,307, Figs 3a,b and Table 1). Pooled meta-analysis across all surveys showed that RDTs detected an average of 41% (95% CI = 26–66%) of PCR-positive infections. This primarily reflects the relationship between RDT and PCR in low-transmission settings, with an average PCR prevalence of 8% across all the clusters included in our analysis.
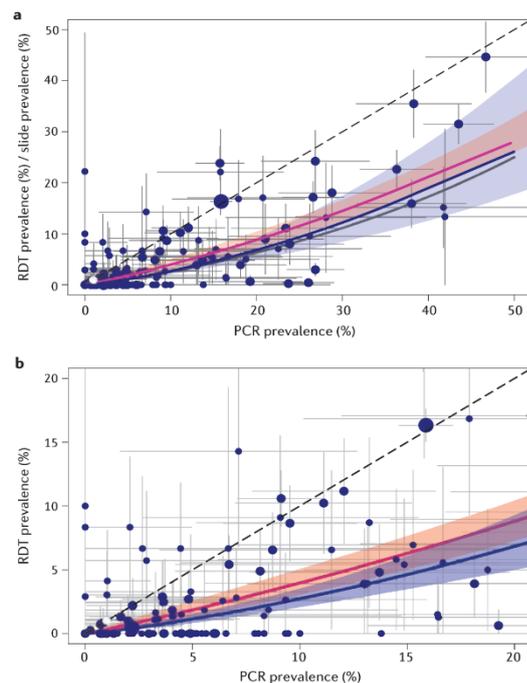
Figure 3 | The relationship between rapid diagnostic test (RDT) and polymerase chain reaction (PCR) prevalence overall (**a**) and zoomed in for <20% PCR prevalence (**b**). Blue, observed RDT:PCR prevalence data and model fit; pink, the triangulated RDT:PCR comparison (see methods); grey, the PCR:microscopy comparison from ref. 13. Dashed lines indicate the expected relationship if RDT (or microscopy) and PCR detected equal prevalence. Horizontal and vertical lines indicate 95% confidence intervals around point estimates, whereas coloured solid lines indicate the median of the Bayesian posterior distributions from the fitted model and shaded areas indicate 95% credible intervals. Radius of point estimates indicate cluster size (from small to large: <100, 100–1,000 and >1,000).

Age, transmission intensity and undetected malaria infection by RDT. As with the relationship between RDT- and microscopy-measured prevalence, stratifying by age group improved the model fit to the data, showing a decrease in detectability by RDT with increasing age (Figs 4a–c). Meta-analysis of the risk ratio between RDT and PCR positivity showed that, for children under 5 years of age, RDTs detected 81% (95% CI = 74–89%) of PCR-positive infections. By comparison, RDTs detected fewer PCR-positive school-aged individuals (6–15 years) (70%, 95% CI = 57–86%), and even fewer among adults over 15 years of age (49%, 95% CI = 31–78%). There was a larger data set available for analysis in the under 5 (140 clusters) and 6–15 (136 clusters) age groups compared with adults (81 clusters), suggesting that additional data in the higher age group could help to improve the accuracy of these estimates.

Previous studies have suggested that the proportion of carriers with sub-microscopic infections decreases in areas of higher transmission intensity, potentially because of an association with re-infection and increased parasite density[13,14]. A similar trend was also observed in the relationship between RDT and PCR detectability. The fit to our data was improved after stratifying by transmission intensity based on PCR-measured prevalence, showing increased RDT sensitivity compared with PCR as transmission increases (Fig. 4d–f). However, meta-analysis of the risk ratio between RDT and PCR positivity did not show a significant difference between the three transmission

ranges, possibly indicating that more data are needed to define a more robust relationship for each transmission setting.

Figure 5 shows RDT detectability as a proportion of PCR-positive individuals, stratified by age and transmission intensity. Irrespective of transmission intensity, adults have the highest percentage of RDT-undetectable infections. By contrast, the percentage of individuals with RDT-detectable infections in all age groups increases as transmission intensity increases. However, since infection rates are greater at high-transmission intensities, RDTs may still miss a larger absolute number of infectious individuals at this level of endemicity. Best-fit model estimates of PCR-measured prevalence based on RDT-measured prevalence are summarized in Figs 3, 4 and Table 1.

Effect of individual-level covariates on RDT:PCR discordance. We evaluated the impact of age and transmission intensity on RDT positivity among PCR-negative individuals as a potential indicator of prolonged HRP2 clearance time. Logistic regression, adjusted for cluster PCR-measured prevalence, showed that among PCR-negative individuals, school-aged children had a significantly higher RDT positivity (OR = 1.53, 95% CI = 1.28–1.82, *p*<0.001) when compared with a baseline of children under 5 years of age. Adults showed similar odds of being RDT positive (OR = 1.00, 95% CI = 0.64–1.58, *p* = 0.990) as those under 5 years. Infections that were undetected by RDT, based on PCR positivity, were highest in adults (OR = 5.04, 95 %CI = 4.14–6.13, *p*<0.001) compared with those under 5 years, with a similar risk in school-aged children and those under 5 years (Supplementary Table 2).

RDT positivity among PCR-negative individuals varied between RDT brands, as did the detection of infection in PCR-positive individuals, but these results were not significant. Patients with a fever were less likely to have undetected infections by RDT if they were PCR positive (OR = 0.14, 95% CI = 0.06–0.32, *p*<0.001), but also more likely to have a RDT-positive result if they were PCR negative (OR = 4.86, 95% CI = 2.29–10.30, *p*<0.001). More recent surveys showed a lower risk of RDT-undetected infections, based on PCR positivity (OR = 0.77 per year, 95% CI = 0.60–0.99, *p* = 0.044), which may indicate an improved performance of RDTs over time. PCR method was associated with test discordance at borderline significance, with RDTs detecting less PCR positive results measured by qPCR than those measured by PCR (OR = 1.92, 95% CI = 0.98–3.74, *p* = 0.056), reflecting higher sensitivity of qPCR, as described previously[15,45].

Model validation. From the leave-one-out analysis, the correlation coefficient between observed and predicted values of RDT-measured prevalence from the RDT:PCR model was 0.67, indicating a moderate agreement. The correlation coefficient between observed and predicted values of RDT-measured prevalence from the RDT:microscopy model was 0.92, indicating a relatively stronger agreement (Fig. 6). The credible interval of this triangulated relationship was narrower than that of the directly observed line, owing to the larger number of data points in the RDT:microscopy and microscopy:PCR data sets (Figs 2, 3, Table 1). There was no significant difference between the triangulated and observed relationships at any transmission intensity.

DISCUSSION

As the burden of malaria continues to decline in many regions[1], it is crucial to understand the suitability of diagnostics for use in low-transmission and near-eliminating areas where MSAT and MDA strategies are likely to be applied. More specifically, how will diagnostic accuracy affect the ability of MSAT programmes to detect and treat asymptomatic individuals or determine local malaria prevalence thresholds for the initiation of MDA? Our study results show that the detection capability of RDTs is comparable with, and often greater than, microscopy. On average, microscopy captured 87% of RDT-positive infections, with higher test concordance in children than in adults. The extent to which this higher RDT detection reflects increased sensitivity, lack of specificity, or both, is unclear. Compared with molecular detection methods, however, RDTs still miss a substantial proportion of infections, capturing only 41% of PCR-positive individuals in low-transmission settings. Our analysis included cross-sectional data with paired prevalence measures by either RDT and microscopy or RDT and PCR from more than 180,000 individuals, spanning more than 400 geographical clusters. The detection levels
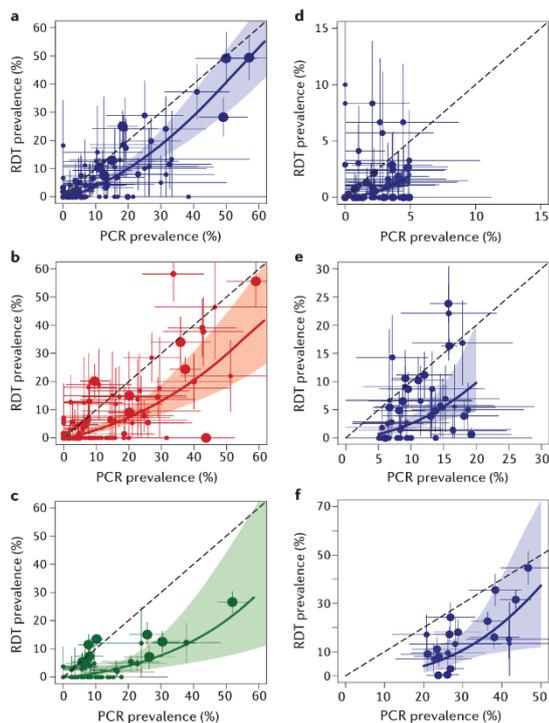
Figure 4 | The relationship between rapid diagnostic test (RDT) and polymerase chain reaction (PCR) prevalence by age group (**a–c**) and PCR prevalence band (**d–f**). The Bayesian model was fitted separately for each age group or PCR prevalence band. Age groups are younger than 5 years (**a**) 6–15 years (**b**) and older than 15 years (**c**). PCR prevalence bands are <5% (**d**), 5–20% (**e**) and >20% (**f**). Dashed lines indicate the expected relationship if RDT and PCR detected equal prevalence. Horizontal and vertical lines around point estimates indicate 95% confidence intervals, whereas coloured solid lines indicate the median of the Bayesian posterior distributions from the fitted model and shaded areas indicate 95% credible intervals of these fits. Radius of point estimates indicate cluster size (from small to large: <50, 50–100 and >100).

observed differed depending on age and transmission intensity, reflecting complex dynamics at both the ecological and host level that may influence parasite densities and the relative performance of these diagnostics.

Factors correlated with the accuracy of RDTs are varied and likely to be driven by subtleties in the concentration and duration of HRP2 antigens in peripheral circulation. A lower specificity by RDT is expected given that, in addition to current infection, they can detect recent infection owing to residual HRP2 even after parasite clearance. Our analysis found that RDTs had a higher positivity rate than microscopy among those who were more likely to have current or recent high parasite densities — children, those with measured or reported fever and those recently treated with antimalarial drugs. This may indicate that high parasite densities and, therefore, ruptured schizonts (asexual parasites that replicate to form multiple red blood cell invading parasites), lead to increased and/or prolonged HRP2 levels. These levels are likely to vary depending on an individual's clinical status and stage of infection owing to associated fluctuations in parasite density. Because RDTs have been designed for clinical use, it is intuitive that their performance would be optimal in the detection of high-density infections associated with symptomatic disease. A previous analysis evaluating the sensitivity of RDTs and microscopy, specifically in
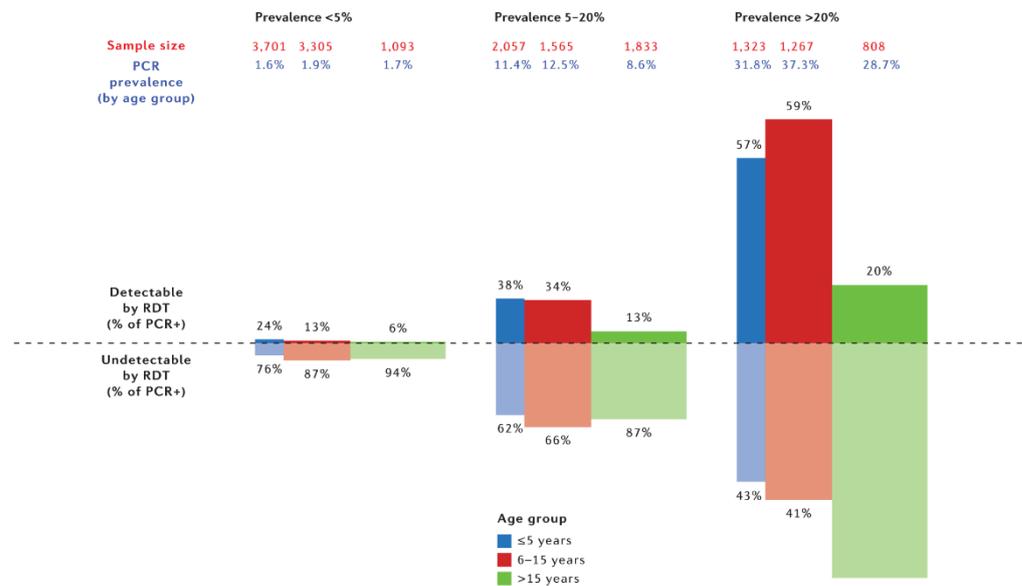
Figure 5 | Rapid diagnostic test (RDT) detectable (darker colours) and undetectable (lighter colours) infections based on polymerase chain reaction positive (PCR+) infections by age (under 5 years, 6–15 years and older than 15 years) and transmission intensity (PCR prevalence <5%, 5–20% and >20%). The height of the bars for RDT detectable and undetectable proportions reflects the total prevalence of infection in that group according to PCR, whereas the width of the bars shows the proportion of the population in each age group in most African settings (younger than 5 years (blue), 15%; 6–15 years (red), 35%; and over 15 years (green), 50% of the total population[70]).

individuals with clinical symptoms, found an association between parasite density and RDT positivity[60]. This study also stressed the issue of false positives and how RDT specificity, in addition to being influenced by parasite density, may be correlated with age and transmission intensity. Further investigation into how RDT accuracy varies between clinical and subclinical populations could help to elucidate the factors that drive these differences. Our analysis also found that using an ITN was associated with better concordance of RDT and microscopy results, most probably due to a lower risk of infection. This distinction is particularly relevant for elimination strategies, because an RDT-positive and microscopy-negative result after parasite clearance may still indicate recent transmission in a population, whereas absence of infection does not. In general, it should be noted that the quality of microscopy is likely to vary more widely than that of RDTs. Microscopy in the context of research surveys is more accurate than those typically encountered during routine surveillance[61]. Therefore, the relative sensitivity of these diagnostics may be more discordant in programmatic settings than the relationship observed in this study.

Our analysis also found a number of factors that correlated with detection by RDT and PCR. Previous studies have demonstrated that the proportion of carriers with sub-microscopic infections decreases in areas of high-transmission intensity, potentially associated with superinfection (new malaria infection in already infected individuals)[13,14]. This trend was also observed in our analysis — the proportion of PCR-measured infections that were detected by RDT increased with higher transmission intensity. Although the interaction between infection, immunity and parasite density in these settings is not fully understood, it has been suggested that only partial cross-immunity is acquired against malaria parasite clones[62]. Greater multiplicity of infection in higher transmission settings could result in higher parasite densities if host immune systems cannot respond to the diversity of parasites or if parasites increase growth rates in the presence of competing clones[14,63]. In addition to transmission intensity, we also observed age-associated variations in RDT detection. Our analysis shows that, after adjusting for transmission intensity, the

odds of having an RDT-undetectable infection in adults was fivefold higher compared with under 5 year olds, potentially owing to more enhanced immune responses in adults that suppress parasite proliferation. This finding coincides well with data that show a lower sensitivity of microscopy relative to PCR among adults[13]. In addition, among PCR-positive individuals, the odds of a positive RDT result was seven times higher in patients with a fever. Overall, these results emphasize that fever, superinfections and childhood infections are commonly associated with high parasite densities, which, in turn, may lead to higher HRP2 levels that persist after parasite clearance. A number of studies have shown a relationship between parasite biomass and HRP2 clearance time[64-66]. However, these studies were predominantly in areas of high-density infections; studies in areas of lower parasite densities are less conclusive[61]. Moreover, HRP2 concentrations may be influenced by duration of infection, parasite sequestration and HRP2 antibody responses[67]. Therefore, characterizing HRP2 detection profiles at parasite densities that are more typically found in elimination settings can help to better gauge the accuracy of RDTs in these areas. Our results also showed that risk of an RDT-positive and PCR-negative test result was higher in school-aged children compared with children under 5 and adults. This may be further evidence for an association between age and recent high parasite density (approximately 2–4 weeks), but may also suggest that infections can fall below the detection limit of PCR and still be captured by RDTs. RDT results that are typically presumed to be false positives may be advantageous when the identification of a recent as well as a current infection is needed, such as in elimination settings, or if HRP2 is still measurable during periods of fluctuating parasite density that drop below the molecular detection threshold. An improved understanding of RDT performance relative to PCR methods of various sensitivities, such as qPCR and LAMP, could help to further benchmark the range at which RDTs can optimally operate. Although the impact of the PCR method on test sensitivity has been investigated in previous studies[14], more data are required to evaluate this relative to RDT sensitivity in more detail.
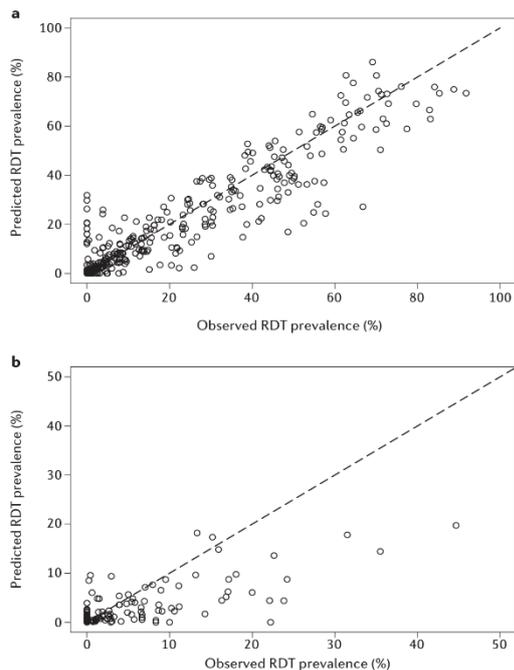
Figure 6 | The relationship between observed and predicted rapid diagnostic test (RDT) prevalence from the RDT:microscopy comparison (**a**), and the RDT:polymerase chain reaction comparison (**b**). Predictions were obtained using leave-one-out cross-validation.

We were able to define a more robust model for the relationship between prevalence measured by RDT compared with microscopy, than for the relationship between prevalence measured by RDT compared with PCR. This is because a more comprehensive data set of comparative RDT and microscopy measures was available across a wider range of transmission intensities. Medium- to high-transmission settings were particularly under-represented in the comparison of RDT and PCR measures. With more than half of our data from <5% PCR prevalence settings (57%; 93 of 162 clusters), the RDT:PCR relationship described here primarily reflects RDT performance at low-transmission intensity. However, the relationship between RDT- and PCR-measured prevalence estimated from directly observed paired data was not statistically different from the RDT:PCR relationship estimated by triangulating the RDT:microscopy and microscopy:PCR relationships based on independent data sets, improving confidence in our findings. Additional covariate information in future studies would further explain other factors that influence diagnostic sensitivity. Although we included RDT brand as a covariate in both the RDT:microscopy and RDT:PCR models, studies in this meta-analysis were not collected specifically to evaluate RDT brand so data are not sufficiently representative to draw conclusions on its impact on diagnostic sensitivity.

Overall, this study has established the relative detection capabilities of existing diagnostics for the identification of asymptomatic individuals infected with *P. falciparum*. To inform community chemotherapy programmes, however, further analysis is needed to determine to what extent these individuals contribute to onward transmission. As with detection, the potential infectiousness of asymptomatic individuals is sensitive to fluctuations in parasite density over the course of an infection and by season[15;68]. These are driven by the maturity of the host's immune response, which may vary by age and by local transmission dynamics, such as seasonality, that can influence population-level

immunity or within-host parasite behaviour. Therefore, defining infectivity in relation to parasite density is especially important; this is addressed further by Slater and colleagues in a companion paper in this supplement[69]. Once the contribution of asymptomatic individuals to the infectious reservoir is better defined, future analyses should ideally establish optimal detection limits of new diagnostics for use in control and elimination strategies.

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SUPPLEMENTARY MATERIAL

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83

Supplementary Tables

Table 1 | Logistic regression analyses of explanatory factors for RDT:microscopy discordance. Adjusted for random effects at the country level.

| | Microscopy+ | | | | | Microscopy- | | | | |
| | RDT-/RDT+ | | | | | RDT+/RDT- | | | | |
	RDT-Mic+/ RDT+Mic+	Crude OR (95% CI)	p	Adjusted[1] OR (95% CI)	p	RDT+Mic-/ RDT-Mic-	Crude OR (95% CI)	p	Adjusted[1] OR (95% CI)	p
Age (years)										
- ≤15	4698/23940	1		1		10493/81372	1		1	
- >15	33/116	1.63 (0.96-2.75)	0.069	1.16 (0.66-2.05)	0.598	105/1229	0.63 (0.31-1.29)	0.205	1.24 (0.73-2.09)	0.426
Gender										
- Female	2488/12910	1		1		5627/43278	1		1	
- Male	2515/12991	1.01 (0.95-1.06)	0.837	1.01 (0.95-1.06)	0.845	5510/42416	1.00 (0.95-1.05)	0.969	0.99 (0.95-1.04)	0.804
Febrile[2]										
- No	1995/9307	1		1		4051/36541	1		1	
- Yes	738/5312	0.59 (0.39-0.89)	0.011	0.55 (0.40-0.76)	<0.001	2907/13901	2.12 (1.58-2.85)	<0.001	1.84 (1.51-2.24)	<0.001
RDT brand										
- Paracheck	2376/12874	1		1		4510/34316	1		1	
- SD-Bioline	2009/9804	1.14 (0.64-2.04)	0.663	0.97 (0.52-1.81)	0.924	5175/34843	1.15 (0.44-3.00)	0.771	1.41 (0.81-2.43)	0.224
- FR	484/2508	1.06 (0.42-2.65)	0.907	1.03 (0.39-2.68)	0.960	1175/8228	1.10 (0.24-5.01)	0.901	0.29 (0.57-2.94)	0.543
- Carestart	95/583	0.86 (0.39-1.90)	0.710	0.50 (0.21-1.20)	0.122	172/6730	0.17 (0.08-0.38)	<0.001	0.43 (0.28-0.65)	<0.001
- ICT	14/29	4.12 (0.83-20.41)	0.082	2.34 (0.42-13.08)	0.334	18/795	0.15 (0.05-0.47)	0.001	0.47 (0.18-1.24)	0.127
ITN use										
- No	2408/13531	1		1		5658/37615	1		1	
- Yes	2526/11964	1.24 (0.98-1.55)	0.068	1.26 (1.00-1.59)	0.053	5369/44611	0.77 (0.64-0.93)	0.006	0.84 (0.73-0.97)	0.019
Antimalarial[3]										
- No	726/4964	1		1		2571/24861	1		1	
- Yes	256/1917	0.90 (0.67-1.20)	0.477	0.90 (0.67-1.20)	0.477	1212/4275	3.43 (2.13-5.51)	<0.001	1.71 (1.16-2.51)	0.006
Year of study		1.12 (0.96-1.31)	0.140	1.08 (0.93-1.24)	0.306		0.95 (0.73-1.23)	0.683	1.06 (0.93-1.20)	0.400
Cluster microscopy prevalence		0.99 (0.98-1.00)	0.004	NA	NA		1.05 (1.04-1.06)	<0.001	NA	NA

RDT: rapid diagnostic test, OR: odds ratio, CI: confidence interval, p: p-value, FR: First Response, ITN: insecticide treated net. [1]Adjusted for cluster microscopy prevalence. [2]Reported fever (DHS) or recorded fever with study-specific cut-off. [3]Self-reported; in the previous 14 days.

Table 2 | Logistic regression analyses of explanatory factors for RDT:PCR discordance. Adjusted for random effects at the study level.

| | PCR+ | | | | | PCR- | | | | |
| | RDT-/RDT+ | | | | | RDT+/RDT- | | | | |
	RDT-PCR+/ RDT+PCR+	Crude OR (95% CI)	p	Adjusted[1] OR (95% CI)	p	RDT+PCR-/ RDT-PCR-	Crude OR (95% CI)	p	Adjusted[1] OR (95% CI)	p
Age (years)										
- ≤5	370/714	1		1		153/6369	1		1	
- 6-15	380/732	1.00 (0.70-1.43)	0.984	0.97 (0.68-1.38)	0.852	190/5407	1.48 (1.29-1.70)	<0.001	1.53 (1.28-1.82)	<0.001
- >15	328/397	4.42 (3.24-6.04)	<0.001	5.04 (4.14-6.13)	<0.001	109/3313	1.38 (0.60-3.18)	0.447	1.00 (0.64-1.58)	0.990
Gender										
- Female	593/970	1		1		265/7562	1		1	
- Male	482/870	0.79 (0.55-1.12)	0.191	0.73 (0.52-1.03)	0.071	187/6647	0.80 (0.59-1.07)	0.132	0.88 (0.71-1.08)	0.226
Febrile[2]										
- No	412/667	1		1		190/2786	1		1	
- Yes	37/206	0.14 (0.06-0.32)	<0.001	0.14 (0.06-0.34)	<0.001	81/308	4.88 (2.31-10.28)	<0.001	4.86 (2.29-10.30)	<0.001
PCR method										
- nPCR	845/1525	1		1		384/7768	1		1	
- qPCR	245/330	2.32 (1.44-3.74)	0.001	1.92 (0.98-3.74)	0.056	68/7334	0.18 (0.06-0.59)	0.005	0.34 (0.10-1.18)	0.090
RDT brand										
- Paracheck	393/575	1		1		169/4275	1		1	
- FR	230/522	0.36 (0.14-0.93)	0.034	0.48 (0.28-0.81)	0.924	131/1500	2.32 (0.62-8.74)	0.212	1.43 (0.55-3.68)	0.460
- Carestart	207/400	0.50 (0.23-1.05)	0.068	0.65 (0.49-0.86)	0.960	74/1607	1.17 (0.25-5.46)	0.839	0.86 (0.34-2.16)	0.742
- ICT	52/70	1.34 (0.30-5.93)	0.702	0.90 (0.17-4.82)	0.122	15/747	0.50 (0.10-2.47)	0.394	0.59 (0.14-2.60)	0.489
- SD-Bioline	62/86	1.20 (0.56-2.54)	0.640	2.07 (1.25-3.43)	0.334	12/140	2.28 (0.49-10.60)	0.294	0.82 (0.46-1.49)	0.526
Year of survey	1855	0.68 (0.54-0.85)	0.001	0.77 (0.60-0.99)	0.044	1502	1.13 (0.71-1.80)	0.595	1.08 (0.78-1.48)	0.654
Cluster PCR prevalence	1855	0.96 (0.93-0.99)	0.014	NA	NA	1502	1.06 (1.03-1.09)	<0.001	NA	NA

PCR: polymerase chain reaction, RDT: rapid diagnostic test, OR: odds ratio, CI: confidence interval, p: p-value, FR: First Response, nPCR: nested PCR, qPCR: quantitative PCR.
[1]Adjusted for cluster PCR prevalence. [2]Recorded temperature ≥37.5°C; collected in 3 out of 9 studies.

Chapter 4: Antibody Responses to Antigenic Targets of Recent Exposure are Associated with Low-Density Parasitemia in Controlled Human *Plasmodium falciparum* Infections

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SECTION A – Student Details

Student	Lotus Leonie van den Hoogen
Principal Supervisor	Chris Drakeley
Thesis Title	The use of antimalarial antibodies to measure transmission in low transmission and pre-elimination settings

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?	Frontiers in Microbiology - Infectious Diseases		
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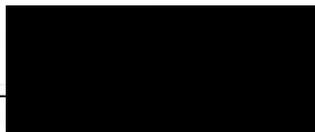
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Antibody Responses to Antigenic Targets of Recent Exposure Are Associated With Low-Density Parasitemia in Controlled Human *Plasmodium falciparum* Infections

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The majority of malaria infections in low transmission settings remain undetectable by conventional diagnostics. A powerful model to identify antibody responses that allow accurate detection of recent exposure to low-density infections is controlled human malaria infection (CHMI) studies in which healthy volunteers are infected with the *Plasmodium* parasite. We aimed to evaluate antibody responses in malaria-naïve volunteers exposed to a single CHMI using a custom-made protein microarray. All participants developed a blood-stage infection with peak parasite densities up to 100 parasites/μl in the majority of participants (50/54), while the remaining four participants had peak densities between 100 and 200 parasites/μl. There was a strong correlation between parasite density and antibody responses associated with the most reactive blood-stage targets 1 month after CHMI (Etramp 5, GLURP-R2, MSP4 and MSP1-19; Spearman's $\rho = 0.82$, $p < 0.001$). Most volunteers developed antibodies against a potential marker of recent exposure: Etramp 5 (37/45, 82%). Our findings justify validation in endemic populations to define a minimum set of antigens needed to detect exposure to natural low-density infections.

Keywords: malaria, antibodies, exposure, controlled human malaria infection (CHMI), sero-surveillance, sero-epidemiology

INTRODUCTION

The use of serological tests to measure antibodies against malaria has been advocated as an adjunct approach to improve the detection of transmission dynamics (Corran et al., 2007; Stewart et al., 2009; malERA Refresh Consultative Panel on Characterising the Reservoir and Measuring Transmission, 2017). This is particularly useful in low transmission settings, where the detection of low-density infections is a major challenge (Okell et al., 2009; Wu et al., 2015). Due to the

longevity of antibody responses they cannot be used as a diagnostic for current infections, but at the population-level, when combined with age, they represent historical and recent transmission (Drakeley et al., 2005; Corran et al., 2007; Stewart et al., 2009). Antibody metrics are less influenced by fluctuations in infection rates between seasons, and where infection rates fall to near elimination, they can help determine whether there is any remaining ongoing transmission. The discovery of antigenic markers that correlate with recent microscopic infection shows promise in the context of detecting recent malaria transmission patterns more sensitively (i.e., up to 1 year) (Helb et al., 2015). However, it remains largely unknown which antigens most reliably induce measurable antibody responses to allow accurate detection of recent exposure to low-density infections.

The identification of antibody responses, and their corresponding antigen targets, following low-density infections in endemic settings is challenging, as the history of previous exposure is often difficult to determine. Longitudinal studies have demonstrated the acquisition of antibodies following asymptomatic infection in endemic areas and suggest that antibodies to some antigens might be more sensitive markers of recent exposure (McCallum et al., 2017). A powerful model to examine this is using controlled human malaria infections (CHMI) in which healthy volunteers are infected via mosquito bites (Roestenberg et al., 2009), parenteral injection with sporozoites (Bastiaens et al., 2016) or infected red blood cells (Pombo et al., 2002). Parasite densities are monitored intensely and remain low as treatment is provided either at the first microscopy-detectable parasitemia, or even earlier at levels detectable only by qPCR (Walk et al., 2016). CHMI studies in non-endemic (reviewed in (Sauerwein et al., 2011)) and endemic (Shekalaghe et al., 2014; Hodgson et al., 2015) settings generally aim to determine correlates of immune protection or test vaccination strategies (Bastiaens et al., 2016). Therefore, responses against mainly pre-erythrocytic antigens have been studied (Felgner et al., 2013; Nahrendorf et al., 2014; Peng et al., 2016), some of which have been suggested as markers of recent parasite exposure (Nahrendorf et al., 2014). However, few have studied antibody responses in previously naïve control groups and only a small number of antigenic targets have been analyzed using enzyme-linked immunosorbent (ELISA) or multiplex bead assays (Turner et al., 2011; Obiero et al., 2015; Hodgson et al., 2016; Burel et al., 2017).

Protein microarrays enable the simultaneous detection of antibody responses to hundreds of antigens to identify biomarkers related to protection or exposure (Boyle et al., 2017). Antigen production for these arrays have mostly used the *in vitro* translation/transcription (IVTT) open reading frame (ORF) method – a polymerase chain reaction (PCR)-based approach that generates large numbers of putative proteins (Davies et al., 2005). In this study, we use a custom-made protein microarray based on purified recombinant malaria antigens which was enriched for antigens associated with recent exposure. Using this array, we aimed to identify immunogenic targets associated with recent low-density *Plasmodium falciparum* infections in previously malaria-naïve CHMI participants.

MATERIALS AND METHODS

Study Population

Fifty-four malaria naïve participants [based on patient history and lack of antibody responses to asexual parasite lysate (Walk et al., 2017)] from eight CHMI studies were included (**Supplementary Table S1**). The study population and sampling frame have been described in detail elsewhere (Bijker et al., 2013, 2014a,b; Bastiaens et al., 2016; Walk et al., 2017; Reuling et al., 2018). In short, volunteers were infected by exposure to five laboratory reared *Anopheles* mosquitoes infected with *P. falciparum* sporozoites of the well characterized NF54 strain, its clone 3D7, or the more recently characterized NF135.C10 (Teirlinck et al., 2013) or NF166.C8 (McCall et al., 2017) strains. Citrate plasma samples for antibody detection were selected at three time points: 1 day pre-challenge (C_{-1}), 21 or 35 days after challenge (median 30 days; C_{+30}) and 64, 140, or 213 days after challenge (median 115 days; C_{+115}). All available samples were analyzed.

Ethics Statement

All clinical trials were carried out in accordance with Good Clinical Practice guidelines and were prospectively registered at ClinicalTrials.gov (NCT numbers listed in **Supplementary Table S1**). All subjects gave written informed consent prior to participation in accordance with the Declaration of Helsinki. Each clinical trial protocol was approved by the Central Committee on Research Involving Human Subjects (CCMO) of the Netherlands (reference numbers listed in **Supplementary Table S1**). Study 8 was also approved by the Western Institutional Review Board (WIRB) in the United States.

Parasite Detection

Volunteers were monitored for the development of symptoms and blood-stage parasites once or twice daily after infection. In studies 1–4 and 8 (**Supplementary Table S1**) parasitemia was treated when detectable by thick blood smear. Blood smears were read according to a standardized protocol for CHMI studies (Bijker et al., 2014a), in which slides are scored as positive if at least two parasites were seen in 0.5 µl of blood (threshold of ∼4 parasites/µl). A second independent microscopist confirmed positivity. In these studies qPCR was performed on all blood samples according to a previously published protocol (Schats et al., 2015). In studies 5–7, qPCR was performed prospectively, and volunteers were treated when parasitemia reached the predetermined threshold of 0.1 parasites/µl (Walk et al., 2016). In study 8, some volunteers had a recrudescent infection after initial subcurative treatment (Reuling et al., 2018).

Protein Microarray

The IgG responses to 40 antigenic targets, all blood-stage related except for one (CSP), were determined using a custom-made protein microarray (see **Supplementary Table S2** for antigen details). Protein preparations at a concentration of 100 µg/µl of protein in printing buffer (ArrayJet, Scotland) were spotted

onto nitrocellulose coated slides (Grace Bio-Labs, United States) with a glycerol-based buffer using the ArrayJet Marathon printer (ArrayJet, Scotland) at the London School of Hygiene and Tropical Medicine (LSHTM). Each slide was sub-divided into 16 arrays with each array consisting of the full complement of antigenic targets printed in duplicate. A standard curve of total human IgG was printed in duplicate within each array (starting concentration 200 µg/ml, fivefold series of 6 points). Samples were processed for IgG detection at the Radboud University Medical Center in Nijmegen. Serum samples were diluted in a deep well at 1:200 in blocking buffer [Phosphate buffered saline (PBS)/BlockIt buffer (ArrayIt Corporation, United States) 25%]. The printed nitrocellulose slides were placed in multi-well hybridization cassettes (HC; ArrayIt Corporation, United States), blocked with 200 µl of blocking buffer and incubated on a rotary shaker (100 rpm) at room temperature (RT) for 1 h. Slides were washed three times: liquid was removed by sharply flicking buffer into a sink, then 200 µl of wash buffer (PBS/Tween 0.05%) was immediately added and the HC placed on the rotary shaker for 2 min. After the final wash, wash buffer was aspirated using a multichannel pipette one column at the time and 100 µl of test samples was added immediately to avoid drying of the nitrocellulose slides. Participant samples, two positive control pools of hyper-immune sera (three repeats of a Ugandan and four repeats of a Tanzanian pool) and one blank (i.e., blocking buffer only) were distributed over twelve slides. Time points from the same participants were grouped on slides where possible to avoid influences of inter-slide variability during assay processing. Slides were incubated for 1 h at RT on the rotary platform. Slides were washed again three times and IgG-specific goat anti-human secondary antibody (Alexa Fluor 633 goat anti-human IgG; Invitrogen) was added in the same manner as the samples at a concentration of 1:1000. Slides were incubated for 1 h at RT on the rotary platform. After a further three washes, slides were dried by centrifuging them at 3000 rpm for 5 min at RT. Slides were stored at $+4°$C and read 3 days after assay processing at LSHTM using the GenePix 4300A scanner (Molecular Devices, United States) at a wavelength of 635 nm.

Median fluorescence intensity (MFI) was background-corrected (i.e., local reactivity around the spot; bkg) and duplicate measurements were averaged (Pearson's correlation coefficient 0.99, $p < 0.001$; **Supplementary Figure S1**). MFI-bkg values smaller than or equal to zero, were replaced with the average value of blank responses and log-transformed. Printing variability was minimal, as determined by the coefficient of variation (CV) of the third point of the standard IgG curve. Inter-slide variability was measured at 1.4% CV, while intra-slide variability was measured at <2.5%. Likewise, assay variability was minimal as shown by the CV of repeated MFI-bkg values of the positive control pools on different slides for GLURP-R2, MSP4 and CSP (associated with high, medium and low antibody responses): 1.5, 0.2, and 5.5% for the Tanzanian pool ($n = 4$), and 0.5, 0.3, and 4.2% for the Ugandan pool ($n = 3$).

Statistical Analyses

All statistical analyses were performed in STATA 14 and PRISM 7. Cumulative parasite density was expressed as the log-transformed area under the curve (AUC) for parasite density versus time in days using the *pkexamine* command in STATA with the *trapezoid* option. Only parasite density results up to, and including, the day of curative antimalarial treatment were included. Tertiles were used to categorize low, medium and high cumulative parasite density. Antibody responses (IgG) were expressed as log-transformed MFI-bkg values. The average response of forty-five participants at C_{-1} plus two standard deviations was used as the threshold for seropositivity by antigen. Antibody responses at C_{+30} and C_{+115} were standardized by subtracting the mean and dividing by the standard deviation (SD) of C_{-1} responses. For both, one outlier at baseline for Etramp 4 Ag 2 was removed (log-transformed MFI-bkg over 8). The Cochran-Armitage test was used to test the trend in the proportion of antigenic targets recognized at each time point over categories of cumulative parasite density. Spearman's rank coefficients (ρ) were used to assess the correlation between antibody responses and cumulative parasite density. The level of statistical significance for individual antigens was adjusted according to the Bonferroni correction. Linear regression was used to test the association between participant characteristics and cumulative parasite density.

RESULTS

Cumulative Parasite Density and Peak Parasite Density

All 54 CHMI participants developed a blood-stage infection after sporozoite-induced challenge through infective mosquito bites [median day of first blood-stage parasites detected by qPCR: 7.0, interquartile range (IQR) 6.5–7.0]. Parasitemias ranged from peak parasite densities below 1 parasites/µl that were treated 7 days post-challenge, to peak densities of 198 parasites/µl that were treated 14 days post-challenge, as well as recrudescent infections that lasted 39 days post-challenge (**Figures 1A–C**). The majority of individuals had peak parasite densities under 100 parasites/µl (50/54), with 19% under one parasite/µl (10/54). A statistically significant difference in cumulative parasite density (expressed as the log-transformed area under the curve for parasite density versus time in days) was seen by gender ($p = 0.011$), which disappeared after adjusting for study ($p = 0.861$). The median age was 21 (IQR 19–22) and did not differ between categories of parasite exposure ($p = 0.541$). As expected, a statistically significant increase was seen in peak parasite density with increasing cumulative parasite density ($p < 0.001$; **Table 1** and **Figure 1D**). Participants with higher cumulative parasite density experienced their peak parasite density later during their infection ($p < 0.001$; **Table 1**).

Minimal Number of Antigens to Detect Infection

The kinetics of IgG responses following challenge are shown in **Figure 2**, while **Figure 3A** shows the number of antigenic targets recognized before and after challenge per category of cumulative parasite density. Two participants recognized more than five out of the panel of 40 antigens pre-challenge (C_{-1}); 1 month post-challenge (C_{+30}) one of these two participants

FIGURE 1 | Parasitemia after controlled human malaria infection over categories of cumulative parasite density **(A–C)** and associated peak parasite densities **(D)**. Tertiles of cumulative parasite density (expressed as the log-transformed area under the curve for parasite density versus time until, and including the day of treatment) were used to categorize low **(A)**, medium **(B)**, and high **(C)**. Peak parasite density is the maximum number of parasites/μl detected during the infection until, and including the day of treatment.

TABLE 1 | Characteristics of the study population according to categories of cumulative parasite density.

	All participants	Categories of cumulative parasite density		
		Low	Medium	High
N	54	19	17	18
Time point[a] (N)				
$-C_{-1}$	45	15	14	16
$-C_{+30}$	45	17	14	14
$-C_{+115}$	32	8	11	13
Cumulative parasite density[b] median (IQR)	8.3 (7.0–9.8)	6.2 (4.6–7.4)	8.7 (8.2–9.2)	10.0 (9.8–10.7)
Peak parasite density[c] median (IQR)	6.9 (2.0–31.6)	1.0 (0.3–2.4)	7.4 (5.1–19.1)	42.6 (22.7–75.4)
Day of peak parasite density median (IQR)	10.4 (7.0–12.3)	7.0 (7.0–9.3)	9.6 (7.0–11.5)	12.2 (10.6–13.5)

Categories of cumulative parasite density are based on tertiles of cumulative parasite density. IQR, interquartile range. [a]Citrate plasma samples for antibody detection were selected at three time points: 1 day pre-challenge (C$_{-1}$), 21 or 35 days after challenge (median 30 days; C$_{+30}$) and 64, 140, or 213 days after challenge (median 115 days; C$_{+115}$). All available samples were analyzed. [b]Cumulative parasite density is expressed as the log-transformed area under the curve for parasites/μl versus time in days until the day of treatment. One participant was not treated until day 36, however, this participant did not have a sample available at C$_{+30}$ for antibody detection thus their total cumulative parasite density was used for categorization in this table. [c]Peak parasite density is the maximum number of parasites/μl during the infection until the day of treatment.

recognized two additional antigens (9 antigens in total) while the other participant recognized thirteen additional antigens (20 antigens in total). For all categories of cumulative parasite density, the peak number of antigens recognized was at C$_{+30}$. The proportion of targets recognized increased over categories of cumulative parasite density ($p < 0.001$ for C$_{+30}$ and 2–7 months post-challenge; C$_{+115}$). High seroprevalence (i.e., over

80%) was seen against GLURP-R2 (91%, 41/45) and Etramp 5 Ag 1 (82%, 37/45) at C$_{+30}$, and against MSP1-19 at C$_{+115}$ (84%, 27/32). For participants with medium to high cumulative parasite density, all were seropositive to GLURP-R2 and 96% to Etramp 5 Ag 1 (27/28) at C$_{+30}$ (**Supplementary Figure S2**), and 96% responded to MSP1-19 at C$_{+115}$ (23/24). For the lowest category, 77% (13/17) responded to GLURP-R2 and 59% (10/17) to Etramp

FIGURE 2 | Kinetics of antimalarial antibody responses after controlled human malaria infection. Antibody responses (expressed as log-transformed median fluorescence intensity corrected for background reactivity) are shown over time, starting 1 day pre-challenge. Red dashed lines represent thresholds of seropositivity using the mean plus two standard deviations of pre-challenge responses across 45 participants. For Etramp 4 Ag 2, the outlier at baseline (blue triangle) was removed for the threshold calculation; the blue dashed line represents the threshold if the outlier at baseline was included.

5 Ag 1 at C_{+30}, and 50% to MSP1-19 at C_{+115} (4/8). Addition of one to two other antigenic targets for this low exposure category at either time point included all participants with an antibody response (see below for one non-responding participant).

Strong Correlation Between Cumulative Parasite Density and Antibody Intensity

Responses up to seven SD greater than the mean of C_{-1} responses were seen at C_{+30} for Etramp 5 Ag 1. Other targets associated with high antibody levels at C_{+30} were GLURP-R2, MSP4 and CSP (SD's greater than five recorded). All participants showed a minimum of one SD greater than the mean of C_{-1} responses for GLURP-R2, while for all other antigens zero to negative responses were seen in at least one of the participants (**Figure 3B**). Highly reactive antigens at C_{+30} were those associated with median responses over arbitrary thresholds of three SD (Top 2: Etramp 5 Ag 1 and GLURP-R2), two SD (Top 4: top 2, as well as MSP4 and MSP1-19) or one SD (Top 10: top 4 as well as GLURP-R0, MSP5, SEA-1, CSP, Etramp 4 Ag 2, AMA1) greater than the mean of C_{-1} responses (**Table 2**). Standardized antibody responses to these top responding antigens were averaged to represent overall antibody density at C_{+30} (**Figure 3C**) and C_{+115} (**Figure 3D**). Most individual antigenic targets showed moderate correlation with parasite exposure (i.e., Spearman's $\rho = 0.50$–0.69) except for GLURP-R0 and CSP (not significant at $p > 0.00125$), while MSP1-19 showed strong correlation (Spearman's $\rho = 0.86$,

$p < 0.001$), see (**Figure 4**). Overall antibody density of top responding antigens showed a strong correlation with cumulative parasite density at C_{+30} (all 40 antigens: Spearman's $\rho = 0.51$, while top 4 responding antigens Spearman's $\rho = 0.82$; $p < 0.001$) and C_{+115} (all 40 antigens: Spearman's $\rho = 0.50$, while top 4 responding antigens Spearman's $\rho = 0.78$; $p < 0.001$).

Participants With Limited Antibody Response

One participant had no demonstrable IgG to any of the 40 malarial antigens tested in samples at either of the post-challenge time points, though tetanus toxoid responses were recorded (log-transformed MFI-bkg values over 7.9 across both time points). For participants with serum samples available at both post-challenge time points, two other participants had an antibody response to only one of the forty antigens at either the C_{+30} or the C_{+115} time point (to CSP or GLURP-R2). The total proportion of individuals with no detectable IgG antibodies at C_{+30} was therefore 4.4% (2/45) and at C_{+115} 6.5% (2/31). These three participants were in the lowest category of cumulative parasite density and had peak parasite densities ≤ 0.20 parasites/µl.

DISCUSSION

Controlled human malaria infection trials provide unique opportunities to study immune responses after exposure

FIGURE 3 | The number of antigenic targets recognized at three time points **(A)**, standardized antibody responses **(B)** and averaged standardized antibody responses **(C,D)**. In **(A,C,D)** results are shown over tertiles of cumulative parasite density (expressed as the log-transformed area under the curve for parasite density versus time until, and including the day of treatment). In **(A)** results are further categorized over time points post-challenge. An antigen is considered recognized if antibody responses (expressed as log-transformed median fluorescence intensity corrected for background reactivity) were higher than the mean plus two standard deviations (SD) of pre-challenge responses across 45 participants (i.e., seropositive). In **(B–D)** antibody responses were standardized by subtracting the mean of pre-challenge responses across 45 participants and dividing by its SD. In **(B)** standardized antibody responses 30 days post-challenge are ordered by median reactivity on the x-axis. Dashed lines represent arbitrary thresholds at 1 SD (red – top 10 antigenic targets), 2 SD (green – top 4 antigenic targets) or 3 SD (blue – top 2 antigenic targets) greater than the mean of pre-challenge responses. In **(C,D)** standardized antibody responses to antigens with a median above these arbitrary thresholds were averaged and are shown 30 days **(C)** and 115 days **(D)** after challenge, using the same color scheme. Average responses across all 40 targets are shown in black.

to a known number of malaria-infected mosquitoes and an accurately quantified parasite exposure (Sauerwein et al., 2011; Scholzen and Sauerwein, 2016). We measured antimalarial antibody (IgG) responses in previously malaria naïve individuals from eight CHMI studies using a custom-made protein microarray. We showed that low parasite densities generated detectable IgG responses in 96% of the participants 1 month after challenge, and in 94% two to 7 months after challenge. Even at the low parasite densities recorded in these participants, a strong correlation was seen between cumulative parasite density and the number of antigenic targets recognized as well as the intensity of IgG responses. Immune responses to a subset of proteins including one hypothesized to be associated with recent exposure were developed by nearly all individuals (i.e., GLURP-R2, MSP1-19 and Etramp 5 Ag 1). It is an important observation that exposure to these infections was detected considering the low parasite density range, which would probably have remained undetected by routine microscopy or rapid diagnostic tests (i.e., 50/54 participants had peak parasite densities <100 parasites/µl while all remained <200 parasites/µl)

(Wongsrichanalai et al., 2007; Okell et al., 2009; Wu et al., 2015).

We assessed IgG reactivity to forty purified recombinant antigens of the *P. falciparum* parasite. All targets were associated with the erythrocytic stage of the parasite life cycle, except for one pre-erythrocytic target (CSP). GLURP-R2, Etramp 5 Ag 1, MSP4 and MSP1-19 were associated with the highest relative antibody responses. Seroprevalence 1 month post-challenge was highest for GLURP-R2 (91%) and Etramp 5 Ag 1 (82%), dropping to approximately two-thirds seropositive two to 7 months post-challenge. Etramp 5 Ag 1 was associated with the highest antibody levels 1 month after challenge relative to pre-challenge responses. This antigen was one of the targets highlighted as a potential marker of recent exposure in a cohort of Ugandan children (Helb et al., 2015). Likewise, GLURP-R2 was associated with recent exposure in a Cambodian population (Kerkhof et al., 2016). Although we were unable to assess the rate of antibody decay in this study due to the small sample size, the low number of individuals with repeated samples and limited follow up time, high reactivity is evident in this previously non-exposed population. Other hypothesized markers of recent exposure

94

TABLE 2 | Characteristics of the top 10 antigenic targets associated with the highest antibody responses 30 days post-challenge in controlled human malaria infection participants.

Gene ID	Description	Name	Allele	Location[a]	AA position	Expression tag	Reference
PF3D7_0532100	Early transcribed membrane protein 5	Etramp 5 Ag 1	3D7	iRBC/PVM	26–111	GST	Spielmann et al., 2003; Tetteh, unpublished
PF3D7_1035300	Glutamate rich protein R2	GLURP-R2	F32	Merozoite (Peripheral)	705–1178	His_{x6}	Theisen et al., 1995
PF3D7_0207000	Merozoite surface protein 4	MSP4	D10	Merozoite surface (GPI-anchored)	43–107	GST	Marshall et al., 1997
PF3D7_0930300	19 kDa fragment of the merozoite surface protein-1	MSP1-19	Wellcome	Merozoite surface (GPI-anchored)	1631–1726	GST	Burghaus and Holder, 1994
PF3D7_0423700	Early transcribed membrane protein 4	Etramp 4 Ag 2	3D7	iRBC/PVM	76–136	GST	Spielmann et al., 2003; Tetteh, unpublished
PF3D7_1035300	Glutamate rich protein R0	GLURP-R0	F32	Merozoite (Peripheral)	94–489	His_{x6}	Theisen et al., 1995
PF3D7_0206900	Merozoite surface protein 5	MSP5	3D7	Merozoite surface (GPI-anchored)	147–207	GST	Marshall et al., 1998
PF3D7_1021800	Schizont egress antigen 1	SEA-1	3D7	Schizont/Maurer's cleft	810–1083	GST	Raj et al., 2014; Tetteh, unpublished
PF3D7_0304600	Circumsporozoite protein	CSP	3D7	Sporozoite surface	20–373	n/a	Kastenmüller et al., 2013
PF3D7_1133400	Apical membrane antigen 1	AMA1	FVO	Sporozoite/Merozoite (Micronemes)	97–546	His_{x6}	Collins et al., 2007

GPI, glycosylphosphatidylinositol; PVM, parasitophorous vacuolar membrane; iRBC, infected red blood cell; GST, glutathione S-transferase; kDa, kilodalton; AA, amino acid. [a]Location information is based on published literature and mass spec proteomic data (plasmodb).

identified by Helb et al. (2015) were also recognized in this population (42% for CSP and 27% for Etramp 4 Ag 2 1 month post-challenge), whereas HSP40 and GEXP18 were not (<5%). At 2–7 months after challenge, MSP1-19 was associated with the highest seroprevalence (84%; 27/32). This target also showed the strongest correlation with cumulative parasite density 1 month post-challenge (Spearman's $\rho = 0.86$, $p < 0.001$). MSP1-19 has been associated with changes in transmission over time (van den Hoogen et al., 2015) and in recent transmission (Supargiyono et al., 2013). MSP4 was the final target in the top four responding antigens 1 month after challenge, while MSP5 was in the top 10. These antigens were associated with protection against clinical disease in Senegalese (Perraut et al., 2017), Brazilian (Medeiros et al., 2013), and Vietnamese (Wang et al., 2001) populations and warrant further investigation. MSP4 was also strongly associated with recent asymptomatic and symptomatic infections in Kenyan children alongside AMA-1, MSP1-19, and EBA140 RII (McCallum et al., 2017).

Previous studies describing antibody responses to malaria in CHMI participants concluded that a single CHMI is sufficient to induce production of antibodies directed against sporozoite, liver-stage and cross-stage antigens (Scholzen and Sauerwein, 2016). In line with our current findings, the magnitude of antibody and memory B-cell responses to cross-stage antigen MSP1-19 was reported to correlate with the degree of parasite exposure (duration and peak density) (Biswas et al., 2014; Elias et al., 2014; Nahrendorf et al., 2014; Walker et al., 2015). The majority of studies examining antibody responses following CHMI focused on antibody responses related to (sterile) protection and identified antigenic targets such as EXP-1 (Obiero et al., 2015), LSA-1 (Felgner et al., 2013; Nahrendorf et al., 2014), TRAP (Peng et al., 2016), and CSP (Felgner

et al., 2013; Nahrendorf et al., 2014; Obiero et al., 2015; Hickey et al., 2016), showing some evidence for an increase in responses with increasing parasite exposure. Only a limited number of studies have reported antibody responses to multiple malarial antigenic targets, other than CSP or AMA1 and MSP1-19 alone, in the previously malaria naïve control groups of CHMI trials (Turner et al., 2011; Obiero et al., 2015; Hodgson et al., 2016). Obiero et al. (2015) and Hodgson et al. (2016) assessed antibody responses after CHMI in both non-endemic and endemic populations using ELISA (Hodgson et al., 2016). Seropositivity against MSP1-19, Rh5, CSP, and LSA-1 were similar to those we recorded. However, seropositivity against AMA-1 was considerably higher (Hodgson et al., 2016) or non-existent (Obiero et al., 2015), compared to moderate responses in our study. These differences may be due to differences in assay protocols, strains used [3D7 (Hodgson et al., 2016) versus FVO] or the levels of parasite exposure. In both studies, antimalarial antibody responses were induced more efficiently in endemic volunteers compared to non-endemic volunteers, even if baseline responses were the same, indicating the presence of memory B cells in endemic populations (Wipasa et al., 2010). Burel et al. (2017) reported that the generation of antibodies to blood-stage antigens following CHMI is strongly influenced by expression patterns of microRNA, which are involved in regulation of immune responses, and that microRNA expression patterns vary considerably between individuals. Turner et al. (2011) explored antibody responses to (multiple regions of) five blood-stage antigenic targets in volunteers from a Dutch CHMI trial using a multiplex bead assay. They found responses in 93% of volunteers 35 days post-challenge, mostly against PfEMP1 (which was not included in our panel) and GLURP-R2, with lower seropositivity against GLURP-R0 and MSP3,

95

FIGURE 4 | The association between cumulative parasite density and standardized antibody responses. Cumulative parasite density is expressed as the log-transformed area under the curve for parasite density versus time until, and including the day of treatment. Antibody responses (expressed as log-transformed MFI corrected for background reactivity) 30 days post-challenge were standardized by subtracting the mean of pre-challenge responses across 45 participants and dividing by its standard deviation (SD). In **(A–J)** Spearman's ρ and associated *p*-values are shown per antigen. Solid lines and shaded areas are fractional polynomial fits with 95% confidence intervals. In **(K,L)** standardized antibody responses against antigenic targets with a median above arbitrary thresholds of 1 SD (red – top 10 antigenic targets), 2 SD (green – top 4 antigenic targets) or 3 SD (blue – top 2 antigenic targets) greater than the mean of pre-challenge responses, were averaged. Average responses across all forty targets are shown in black. In **(K)** Spearman's ρ for all 40: 0.51, top 10: 0.77, top 4: 0.82, top 2: 0.61; *p* < 0.001 and in **(L)** all 40: 0.50, top 10: 0.69, top 4: 0.78, top 2: 0.72; *p* < 0.001.

similar to our results. They saw no association between PfEMP1 antibody acquisition and parasite load or maximum parasite density (Turner et al., 2011). The strong correlations seen in the current cohort are most likely due to the increased range in parasite exposure. In addition to the overlap in these previously described results, we show responses to a range of erythrocytic antigens including those potentially associated with recent infection using a newly developed custom-made protein microarray.

Studying antibody responses following malaria infections in volunteers from non-endemic areas is informative due to difficulties in assessing the exact level and frequency of previous exposure in endemic populations. Genetic and environmental differences between malaria endemic and non-endemic populations make direct translation of these results challenging. Moreover, parasite densities are likely to reach considerably higher levels in natural infections, which in turn would influence the number of antigenic targets recognized and the intensity of existing responses. Nevertheless, an overlap was seen between our results and those from endemic populations for certain antigenic targets [Etramp 5 (Helb et al., 2015), Etramp

4 (Helb et al., 2015), CSP (Baum et al., 2013; Helb et al., 2015), MSP4 (Baum et al., 2013; Burel et al., 2017; McCallum et al., 2017), MSP1-19 (McCallum et al., 2017) and to a lesser extent in our results AMA-1 (Stanisic et al., 2015; McCallum et al., 2017)], while not for other targets [GEXP18 (Helb et al., 2015), HSP40 (Helb et al., 2015), EBA140, EBA175, and MSP2 (Stanisic et al., 2015; Burel et al., 2017; McCallum et al., 2017)]. Overall, it is an important observation that antibody responses could be detected in a previously non-exposed population following such low-density infections. The duration of detectable antibody titres in the current study population, especially after re-infection, is unknown. This information is of use for validation of these targets in sero-surveillance aiming to generate proxy estimates of incidence for transmission monitoring. Further assessment of antibody kinetics following infection (i.e., assessing time since infection) using these antigenic targets is essential. Although all participants were previously naïve for malaria, we saw reactivity to some of the included targets at the pre-challenge time point (i.e., Etramp 4 Ag 2) potentially due to cross-reactivity with antigens from other pathogens. Likewise, responses to AMA1 and MSP1-19 were seen in some of the malaria naïve United Kingdom

adults pre-challenge at similar concentrations to those detected at C_{+30} (Hodgson et al., 2016). Using a two-standard deviation rule to define the threshold of positivity has its limitations; by default, this will cause some participants to be defined as seropositive pre-challenge (i.e., approximately 2.5%). One participant remained undetected for IgG responses against the panel of 40 antigens evaluated. This may be due to the very low exposure to malaria parasites as their peak parasite density was the lowest recorded (0.15 parasites/μl) and the duration of their infection as detected by qPCR was 1 day. Two other participants with limited antibody responses recorded (i.e., to one of the antigens in the panel at only one of the time points) had peak parasite densities of ≤0.20 parasites/μl. Furthermore, the panel of targets evaluated is finite and there may be antigens not yet expressed that would have induced a detectable immune response in these participants. Lastly, IgM responses were not evaluated in this study, which may also have been present in these participants.

CONCLUSION

Antibody responses to erythrocytic antigens were detectable following low-density experimental *P. falciparum* infections in nearly all volunteers. This included antigenic targets potentially related to recent infection (Helb et al., 2015) as well as well-known targets such as AMA1, MSP1-19, and CSP. Detecting exposure to recent infections below the detection limit of conventional diagnostics is essential to interrupt transmission (Ouédraogo et al., 2016; malERA Refresh Consultative Panel on Characterising the Reservoir and Measuring Transmission, 2017), especially in low transmission and elimination settings where the majority of infections are of low-density (Okell et al., 2009; Wu et al., 2015). We showed a strong dose-response relationship between cumulative parasite density and antibody density across multiple targets. Moving forward, a selection of 4–5 targets could be combined in a field-based assay such as an ELISA to rapidly assess remaining transmission in (near-) eliminating settings. This would be advantageous compared to measuring infection rates, as PCR-based techniques are more costly and labor-intensive, require larger sample sizes in low transmission settings, are more sensitive to fluctuations in parasite densities during an infection and overall rates between seasons. Ultimately, our findings require validation in endemic populations to define the minimum set of antigens needed to reliably detect exposure to natural infections.

AUTHOR CONTRIBUTIONS

LH, JW, TO, IR, TB, CD, RS, and KT designed the study. JW, IR, TB, and RS were involved in the design and performance of the original CHMI studies, and collection of all samples. LR, JB, RC, SS, SD, and KT provided antigen constructs. TO and KT designed the assay. LH and TO processed samples. LH, JW, IR, TB, CD, and KT performed data analyses and interpreted results. LH drafted the manuscript with support from JW, TO, IR, JB, TB, CD, RS, and KT. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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98

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

99

Supplementary Tables

Supplementary Table 1: Controlled human malaria infection trials included in the study. Samples from participants across eight controlled human malaria infection trials were analysed in the current study.

Study	Year	NCT number	Ethical approval CCMO	Reference
1	2011	NCT01236612	NL34273.091.10	[24]
2	2011	NCT01218893	NL33904.091.10	[25]
3	2012	NCT01422954	NL 37563.058.11	[26]
4	2012	NCT01728701	NL39541.091.12	[9]
5	2015	NCT02080026	NL48301.091.14	-
6	2015	NCT02098590	NL48732.091.14	[23]
7	2015	NCT02098590	NL48732.091.14	[23]
8	2016	NCT02836002	NL56659.091.16	[27]

NCT: National Clinical Trial. CCMO: Central Committee on Research Involving Human Subjects.

Supplementary Table 2: Characteristics of antigens included on the protein microarray.

Order	Gene ID	Acronym	Description	Location	Expression tag	Strain	Reference
1	PF3D7_1001100_1_ACBP1	ACBP1	exported protein, unknown function: Phista	iRBC	GST	3D7	Helb et al (PMID: 26216993); Tetteh unpublished
2	PF3D7_0731600	ACS5 Ag 4	Acyl CoA Synthase. Antigen 4 (fragment 4)	iRBC	GST	3D7	Helb et al (PMID: 26216993); Tetteh unpublished
3	PF3D7_0304600	CSP	Most predominant and antigenic protein on sporozoite surface. Component of RTS,S vaccine	Sporozoite	n/a	3D7	Kastenmuller et al (PMID: 23275094)
4	PF3D7_1301600	EBA140 RIII-V	erythrocyte binding antigen 140; involved in invasion	Apical organelles, micronemes	GST	3D7	Richards et al (PMID: 20843207)
5	PF3D7_0731500	EBA175RII_F2	erythrocyte binding antigen 175; RBC binding region via glycophorin A	Apical tip	GST	3D7	Richards et al (PMID: 20843207)
6	PF3D7_1101800	EPF1v2	Exported protein; PV to iRBC surface	iRBC, maurer's cleft	GST	3D7	Helb et al (PMID: 26216993); Tetteh unpublished
7	PF3D7_0423700	Etramp 4 antigen 1	Early transcribed membrane antigen. Integral PVM protein. N-terminal	iRBC, PVM	GST	3D7	Helb et al (PMID: 26216993); Tetteh unpublished
8	PF3D7_0423700	Etramp 4 antigen 2	Early transcribed membrane antigen. Integral PVM protein. C-terminal	iRBC, PVM	GST	3D7	Helb et al (PMID: 26216993); Tetteh unpublished
9	PF3D7_0532100	Etramp 5 Ag 1	Early transcribed membrane antigen. Integral PVM protein.	iRBC, PVM	GST	3D7	Spielmann et al (PMID: 12686607); Tetteh K unpublished

Order	Gene ID	Acronym	Description	Location	Expression tag	Strain	Reference
10	PF3D7_0532100	Etramp 5 Ag 2	Early transcribed membrane antigen. Integral PVM protein.	iRBC, PVM	GST	3D7	Helb et al (PMID: 26216993); Tetteh unpublished
11	PF3D7_0402400	GEXP18	Gametocyte exported protein 18. Unknown function.	iRBC/Gametocyte	GST	3D7	Helb et al (PMID: 26216993); Tetteh unpublished
12	PF3D7_1035300	GLURP R0	Glutamate rich protein R0	Merozoite Surface	n/a	F32	Theisen et al (PMID: 7719909)
13	PF3D7_1035300	GLURP R2	Glutamate rich protein R2	Merozoite Surface	n/a	F32	Theisen et al (PMID: 7719909)
14	PF3D7_0501100.1	HSP40 Ag 1	Heat shock protein 40	iRBC	GST	3D7	Helb et al (PMID: 26216993); Tetteh unpublished
15	PF3D7_0501100.1	HSP40 Ag 3	Heat shock protein 40	iRBC	GST	3D7	Helb et al (PMID: 26216993); Tetteh unpublished
16	PF3D7_0207000	MSP4	Merozoite surface protein.	Merozoite surface	GST	D10	Marshall et al (PMID: 9353020)
17	PF3D7_0206900	MSP5	Merozoite surface protein.	Merozoite surface	GST	3D7	Marshall et al (PMID: 9719507)
18	PF3D7_1035700	MSP DBL1 N-term	MSPDBL1 N-terminal	Merozoite surface	GST	3D7	Tetteh et al (PMID: 23897617)
19	PF3D7_0930300	MSP1 bk2 MAD20 Rep	MSP1 block 2 MAD20 allele (MAD20 type) repeat region only.	Merozoite surface	GST	MAD20	Polley et al (PMID: 12654798)
20	PF3D7_0930300	MSP1 bk2 MAD20 full	MSP1 block 2 MAD20 allele (MAD20 type) full-length region (repeat and type-specific flanking region).	Merozoite surface	GST	MAD20	Kimbi et al (PMID: 15109551)

Order	Gene ID	Acronym	Description	Location	Expression tag	Strain	Reference
21	PF3D7_0930300	MSP1 bk2 Well Rep	MSP1 block 2 Wellcome allele (MAD20 type) full block 2 region, repeat only, type specific flanking region removed	Merozoite surface	GST	Wellcome	Polley et al (PMID: 12654798)
22	PF3D7_0930300	MSP1 block 2 Palo Alto Repeat	MSP1 block 2 Palo Alto allele K1 type) full block 2 region, repeat only, type specific flanking region removed	Merozoite surface	GST	Palo Alto	Polley et al (PMID: 12654798)
23	PF3D7_0930300	MSP1 block 2 Palo Alto full #6	MSP1 block 2 Palo Alto allele K1 type) full block 2 region, includes type specific flanking region	Merozoite surface	GST	Palo Alto	Polley et al (PMID: 12654798)
24	PF3D7_0930300	MSP1 block 2 R033	MSP1 block 2 R033 allele (R033 type) full-length.	Merozoite surface	GST	RO33	Kimbi et al (PMID: 15109551)
25	PF3D7_0930300	MSP1 block 2 Well full	MSP1 block 2 Wellcome allele (MAD20 type) full block 2 region, includes type specific flanking region	Merozoite surface	GST	Wellcome	Kimbi et al (PMID: 15109551)
26	PF3D7_0206800	MSP2 CH150/9	CH150/9 allele of MSP2. Full-length.	Merozoite surface	GST	CH150/9	Polley et al (PMID: 16111789)
27	PF3D7_0206800	MSP2 Dd2	Dd2 allele of MSP2. Full-length.	Merozoite surface	GST	DD2	Taylor et al (PMID: 7591074)
28	PF3D7_1035400	MSP3-3D7	MSP3 3D7 allele	Merozoite surface	MBP	3D7	Polley et al (PMID: 17191173)
29	PF3D7_1035700	PF10_0348 C-term	MSPDBL1 C-terminal	Merozoite surface	GST	3D7	Tetteh et al (PMID: 23897617)
30	PF3D7_1036300	PF10_0355 C-term	Duplicate antigen. MSPDBL2 C-terminal	Merozoite surface	GST	3D7	Tetteh et al (PMID: 23897617)

Order	Gene ID	Acronym	Description	Location	Expression tag	Strain	Reference
31	Pf3D7_0402000	Phista_0402000	exported protein, unknown function: Phista	iRBC cytoplasm	GST	3D7	Helb et al (PMID: 26216993); Tetteh unpublished
32	Pf3D7_0424900	Phista_0424900	exported protein, unknown function: Phista	iRBC cytoplasm	GST	3D7	Helb et al (PMID: 26216993); Tetteh unpublished
33	Pf3D7_0425400	Phista_0425400	exported protein, unknown function: Phista	iRBC cytoplasm	GST	3D7	Helb et al (PMID: 26216993); Tetteh unpublished
34	Pf3D7_0601700	Phista_0601700	exported protein, unknown function: Phista	iRBC cytoplasm	GST	3D7	Helb et al (PMID: 26216993); Tetteh unpublished
35	PF3D7_1133400	AMA1	Apical membrane antigen 1	micronemes	His	FVO	Collins et al (PMID: 17192270)
36	PF3D7_0930300	MSP1-19	19kDa fragment of MSP1 molecule.	Merozoite surface	GST	Wellcome	Burghaus and Holder (PMID: 8078519)
37	PF3D7_1021800	PfSEA-1	Schizont egress antigen.	iRBC	GST	3D7	Raj et al (PMID: 24855263); Tetteh K unpublished
38	PF3D7_0936300	REX 3	Ring exported protein,	iRBC	GST	3D7	Helb et al (PMID: 26216993); Tetteh unpublished
39	PF3D7_0424100	Rh5	Receptor for human protein Basigin.	Apical tip	C-tag	3D7	Jin et al (PMID: 30131879); Crosnier et al (PMID: 22080952); Tetteh K unpublished

Order	Gene ID	Acronym	Description	Location	Expression tag	Strain	Reference
40	PF3D7_0501300	SBP1	skeleton-binding protein; essential for translocation of PfEMP1 to iRBS surface via Maurer's cleft.	iRBC	GST	3D7	Gruring et al (PMID: 21266965); Tetteh K unpublished

GPI: Glycosylphosphatidylinisotol; PVM: parasitophorous vacuolar membrane; iRBC = infected red blood cell.

Supplementary Figures
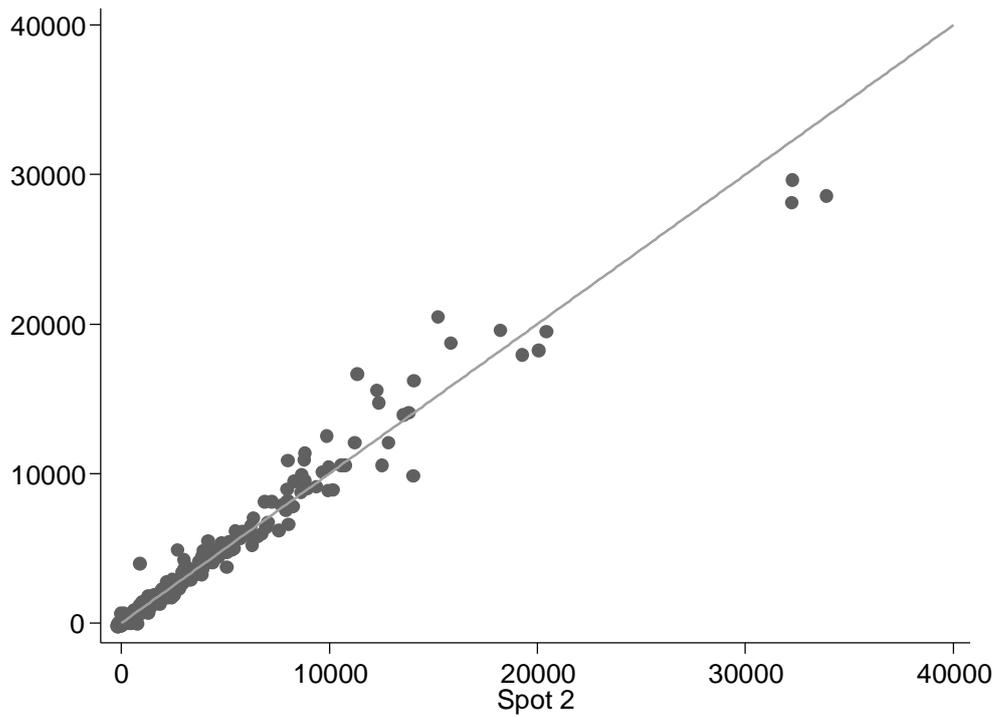
Supplementary Figure 1: Duplicate median fluorescence intensity measures from repeated spots on the protein micro-array. Paired measurements across all the samples tested are shown; duplicate measures were averaged for analyses. The grey line represents the line of equality. Pearson's ρ=0.99, p<0.001.
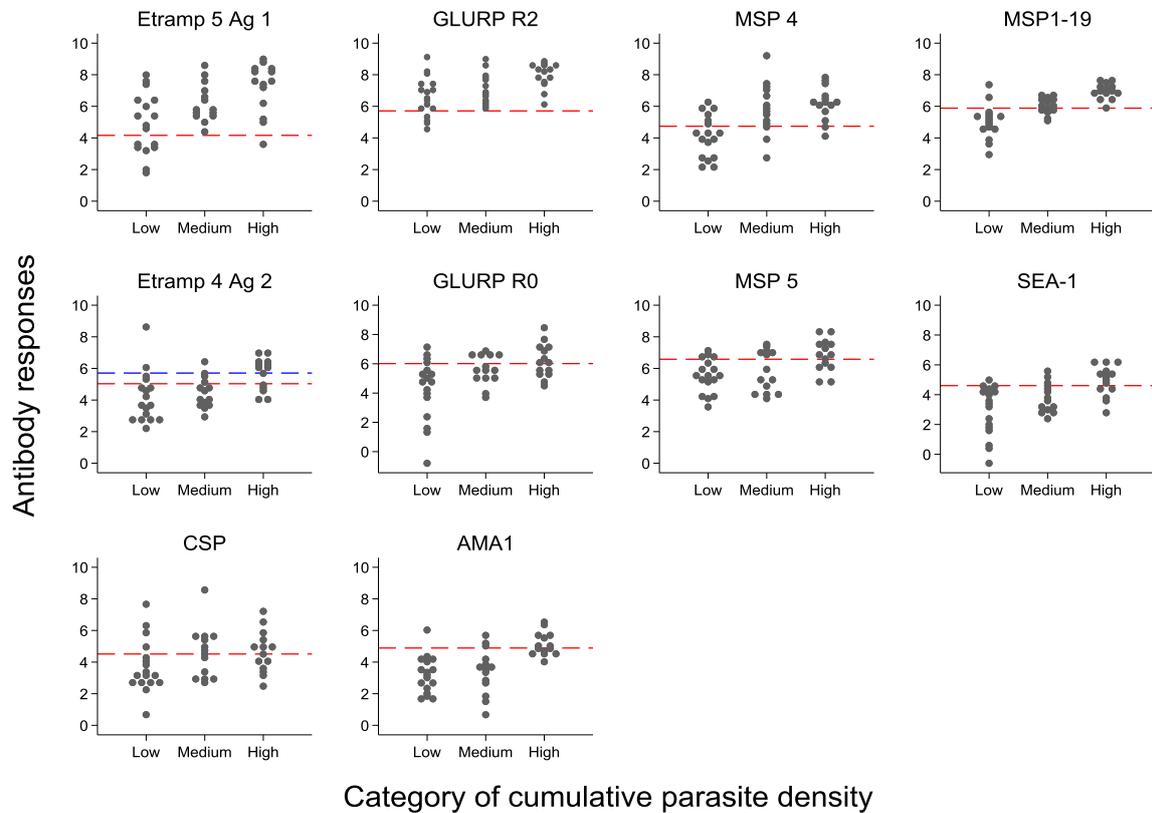
Supplementary Figure 2: Antibody responses over categories of cumulative parasite density thirty days post-challenge. Cumulative parasite density is expressed as the log-transformed area under the curve for parasite density versus time until, and including the day of treatment. Tertiles were used to categorise low, medium and high. Antibody responses are expressed as log-transformed median fluorescence intensity corrected for background reactivity. Red dashed lines represent thresholds of seropositivity using the mean plus two standard deviations of pre-challenge responses across 45 participants. For Etramp 4 Ag 2, the outlier at baseline was removed for the threshold calculation; the blue dashed line represents the threshold if the outlier at baseline was included.

Additional File: Comparison of median fluorescence intensity measures recorded by protein microarray and multiplex bead assay

To compare median fluorescence intensity (MFI) measures for antimalarial IgG recorded by protein microarray and suspension bead array (SBA, synonym for MBA), samples from controlled human malaria infection (CHMI) participants were tested on both platforms. Samples were available at one day pre-CHMI, thirty days post challenge or 2-7 months post-CHMI. The thirty days post-CHMI time point was chosen as the highest antibody responses were recorded at this time point by protein microarray (**Chapter 4**, page 93).

Methods

The assay protocol for protein microarray was performed as described in this chapter (**Chapter 4**, page 90). For SBA this was performed as described by Wu et al. (*in press*, Wellcome Open Research). Serum samples were tested for antimalarial IgG at a serum concentration of 1:200 for both platforms. All the antigens that were included on the protein microarray and optimised for use on the SBA platform at the time were analysed (8 out of 40 antigens analysed on the protein microarray in **Chapter 4**). For antigen abbreviations see Supplementary Table 2 (**Chapter 4**). All statistical analyses were performed in STATA 14.

Results

Figure 1 shows paired MFI measurements as recorded by protein microarray and SBA in 45 CHMI participant samples thirty days post-CHMI. Generally, absolute values for MFI are higher on the SBA compared to protein microarray, except for CSP and SEA-1. Strong correlation (i.e. correlation coefficient >0.7) was seen in recorded MFI values on both platforms (Table 1), except for AMA-1 which showed moderate linear correlation (Pearson's correlation coefficient 0.539, $p<0.001$). In addition, both CSP and AMA-1 showed moderate rank correlation (Spearman's correlation coefficient 0.647 and 0.540, respectively).

Conclusion

A strong correlation was recorded between MFI measurements recorded by protein microarray and SBA in CHMI participants thirty days post-CHMI for six out of eight antigens analysed (Table 1). Antibody responses in CHMI participants are likely to be lower than those recorded following naturally acquired infections as parasite densities remain low (i.e. peak parasite densities remained below 200 parasites/µl for all participants). Therefore, antibody responses for some antigens remained low (e.g. AMA-1) thus making it difficult to draw conclusions on the agreement between platforms for these

antigenic targets. Discordances in MFI measures between platforms may have been caused by variations in immunogenicity of antigen batches, differences in antigen concentrations, improved conservation of the conformational structure of antigens on beads (SBA) compared to nitrocellulose microscope slides (protein microarray), or other assay-specific factors (i.e. the fluidics of antigen-antibody interaction in SBA compared to fixation of antigen to slides in protein microarray).

Future work should focus on comparing MFI measurements between protein microarray and SBA/MBA using samples with a wider range of antibody responses as the current analyses only included samples from individuals who harboured low-density infections. Therefore, the current analyses gave limited information for those antigenic targets that this population did not show a response to (e.g. AMA-1 which showed the lowest Spearman correlation coefficient: 0.540). Technically the MBA platform can be assumed the gold standard in this comparison as antigen concentrations for bead couplings are optimised through titration against a standard, whereas protein microarray uses a set amount of antigen printed on the slide (i.e.100 µg/µl). Moreover, as mentioned, the conformational structure of antigens is more likely to be conserved in SBA and the fluidics of the SBA assay allows efficient interaction of antigens and antibodies.
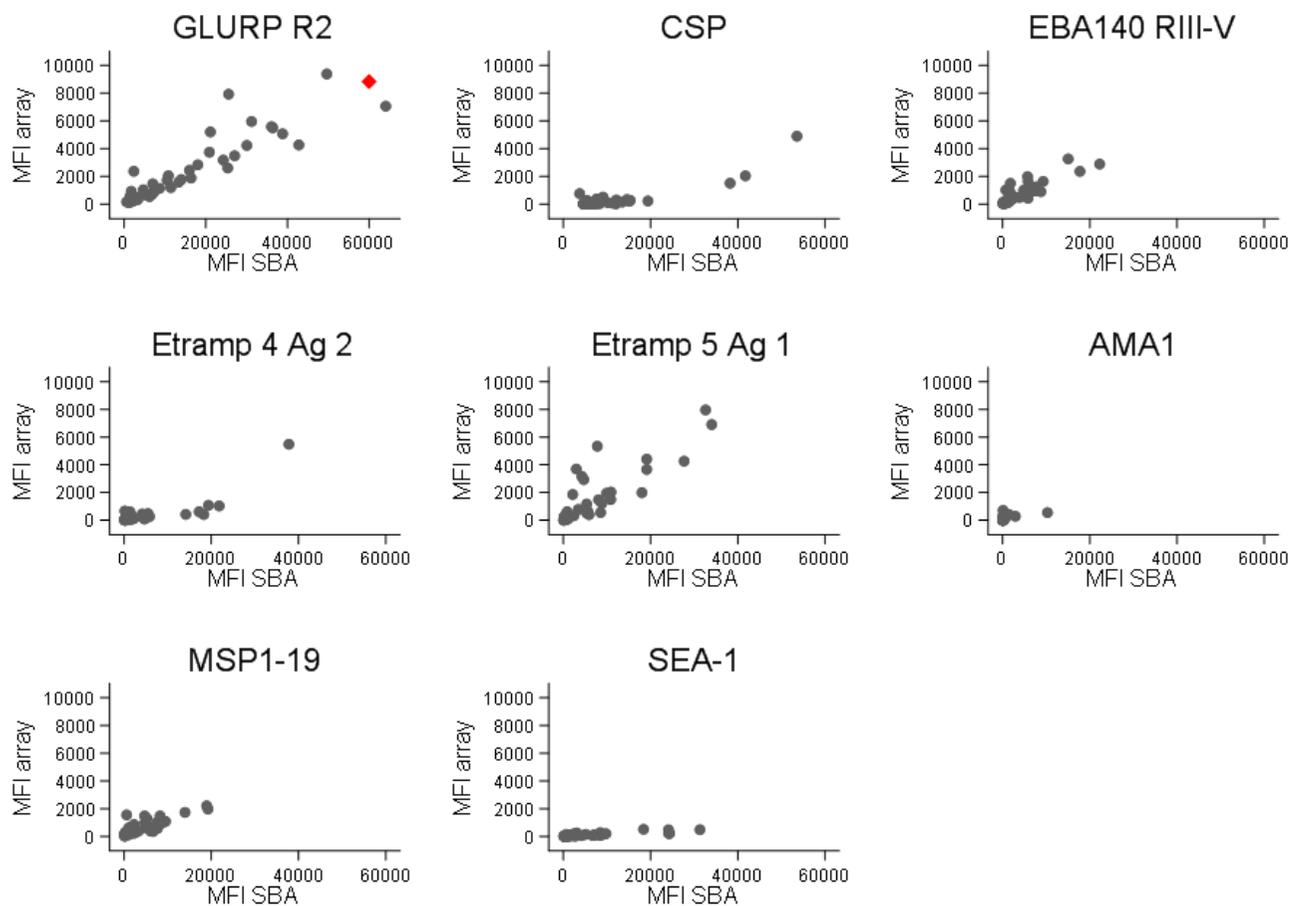
Figure 1: Comparison of median fluorescence intensity (MFI) measured by protein microarray (array) and suspension bead array (SBA; i.e. multiplex bead assay) in controlled human malaria infection participants thirty days post-challenge. Samples were available for 45 participants. Participant samples are shown in grey dots, while the red marker in the left top plot represents a pool of hyperimmune Tanzanian sera. For antigen abbreviations, see Supplementary Table 2 (**Chapter 4**).

Table 1: Correlation coefficients for paired median fluorescence intensity measurements recorded by protein microarray and suspension bead array (i.e. multiplex bead assay) in samples from controlled human malaria infection participants thirty days post-challenge. Samples were available for 45 participants.

Antigen	Pearson's correlation coefficient	p-value	Spearman's correlation coefficient	p-value
GLURP-R2	0.899	<0.001	0.925	<0.001
CSP	0.889	<0.001	0.647	<0.001
EBA140 RIII-V	0.884	<0.001	0.892	<0.001
Etramp 4 Ag 2	0.817	<0.001	0.725	<0.001
Etramp 5 Ag 1	0.855	<0.001	0.872	<0.001
AMA-1	0.539	<0.001	0.540	<0.001
MSP-1$_{19}$	0.822	<0.001	0.834	<0.001
SEA-1	0.825	<0.001	0.814	<0.001

Chapter 5: Application and Quality Control of Multiplex Antibody Detection for Malaria Transmission Surveys

RESEARCH PAPER COVER SHEET

PLEASE NOTE THAT A COVER SHEET MUST BE COMPLETED FOR EACH RESEARCH PAPER INCLUDED IN A THESIS.

SECTION A – Student Details

Student	Lotus Leonie van den Hoogen
Principal Supervisor	Chris Drakeley
Thesis Title	The use of antimalarial antibodies to measure transmission in low transmission and pre-elimination settings

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?			
When was the work published?			
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion			
Have you retained the copyright for the work?*	Choose an item.	Was the work subject to academic peer review?	Choose an item.

*If yes, please attach evidence of retention. If no, or if the work is being included in its published format, please attach evidence of permission from the copyright holder (publisher or other author) to include this work.

SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	Malaria Journal
Please list the paper's authors in the intended authorship order:	Lotus L. van den Hoogen, Jacquelin Présumé, Ithamare Romilus, Gina Mondélus, Tamara Elismé, Gillian Stresman, Thomas Druetz, Ruth Ashton, Vena Joseph, Thomas P. Eisele, Karen Hamre, Michelle Chang, Jean Frantz Lemoine, Kevin K.A. Tetteh, Jacques Boncy, Alexandre Existe, Chris Drakeley & Eric Rogier
Stage of publication	**Not yet submitted**

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I supported training of laboratory staff, conducted data analyses and wrote the first draft of the manuscript with support from ER and CD.

Student Signature: ▮ Date: 28/1/2019

Supervisor Signature: ▮ Date: 25/1/2019

114

Application and quality control of multiplex antibody detection for malaria transmission surveys

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Abstract

Background:

Measuring antibody responses to malaria can aid in describing malaria transmission patterns especially at low transmission where infections and cases are infrequent. A pre-requisite to this is a standardised assay to adequately compare antibody measures between surveys and populations. Here we describe the in-country application and retrospective quality control of a multiplex bead assay (MBA) used to collect antibody measurement in large-scale malaria transmission surveys in Haiti.

Results:

Antibody measurements (IgG) to twenty-one recombinant antigens and peptides were collected for 32,758 participant samples in eighteen weeks using a recently described One-Step protocol (in which test sample and anti-human IgG are incubated simultaneously). Standard curves of a pool of hyperimmune sera from Haitians as well as a *Plasmodium falciparum* WHO reference standard (10/198) were tested with samples. 5-parameter logistic regression was used to fit the sigmoidal relationship between dilution points of hyperimmune sera pools and recorded antibody measures. Inspection of the median and interquartile range (IQR) for the y-inflection point of standard curves was used to determine assay precision within and between surveys. Median and IQRs were similar for Survey 1 and Survey 2 for most antigens, while the length of the IQR for y-inflection points increased for some antigens in Survey 3. The sigmoidal relationship between paired measurements of 804 samples on the One-Step protocol and a Stepwise protocol (in which sample and anti-human IgG were incubated separately with washes in between assay steps) was used to transform One-Step responses to Stepwise responses for all survey samples per antigen. The performance of this transformation was confirmed by the strong correlation of transformed responses compared to Stepwise responses (Pearson's correlation coefficients ranged from 0.68 to 0.95 depending on the antigen).

Conclusion:

This study described the successful in-country application of the MBA with high throughput and acceptable inter-plate variability in Haiti. The highly efficient collection of antibody responses (IgG) allows for rapid assessment of the exposure history of populations which can directly inform control and elimination policies.

Key words: Antibody detection, multiplex bead assay, Luminex, sero-surveillance, sero-epidemiology.

Background

Measurement of antibody responses to malaria at the population-level can describe recent and historical transmission patterns [1–4] and are informative for malaria research and control policies [5–7]. Antibodies can be measured by a variety of techniques including the enzyme-linked immunosorbent assay (ELISA) and multiplex bead assays (MBA). The latter allows the simultaneous detection of antibodies to multiple antigenic targets and have first been described for *Plasmodium* in 2006 [8]. Since then numerous assay optimisation and implementation studies [9–17] as well as epidemiological application studies [18–21] have been published.

Whilst MBA's have the advantage of reduced need of reagent quantities, sample volume and technician time compared to the ELISA in generating responses to many antigens simultaneously [8,9], the multiplex nature is not without technical challenges. All assays will require standards to assess variability across runs or batches, and to compare research studies. With a broad panel of antigens in the MBA it is potentially difficult to find standards for all targets in the panel. Recently, standard curves of known concentrations of total human IgG have been suggested which would enable standardisation [13]. However, these did not show sufficient reproducibility between operators. Moreover, these do not allow for the assessment of antigen-specific responses which are important for quality control in assessing the stability of antigens. Pools of hyperimmune sera are commonly used to identify the dynamic range of responses to antigen panels. Recently, a reference *Plasmodium falciparum* (*Pf*) WHO standard lyophilised plasma standard has been produced [22], and applied in a MBA against a panel of 40 malarial and non-malarial antigens [15].

Previous studies on the application and validation of the MBA have shown its correlation with ELISA [8,10,12,17,23], the stability and reproducibility of coupled beads [10,12,16], the use of Ig subclasses [15] and IgG isotypes [11,15] as well as mono- vs. multiplex results [10,12,15–17]. Although intra- and inter-assay variability have been discussed [10,13,14,16,24] as well as analytical methodologies to determine inter-assay variability [25,26], few have formally assessed this on a large-scale (studies of several thousand samples over time) [16]. Rogier et al. recently described a One-Step MBA protocol in which sample and secondary are incubated simultaneously which further increases the ease-of-use and throughput of the MBA (Rogier et al., *in prep*). Here, we discuss the application and retrospective quality control process of the One-Step MBA protocol for antimalarial antibody (IgG) detection in large-scale malaria transmission surveys in Haiti.

Methods

Study population

Three cross-sectional surveys were conducted: two in the Artibonite valley, central Haiti (Survey 1 in May-Jun 2017 and Survey 2 in Jul-Sep 2017 with a two-week pause due to hurricanes) and one in Grand'Anse, south-western Haiti (Survey 3 in Nov-Dec 2017). Survey 1 included 6,006 participants, Survey 2 21,891 and Survey 3 5,034. In the former two surveys finger-prick blood was collected in microtubes and spotted on Whatmann 903 cards at the end of the day using pipettes (60 µl per spot), whereas in the latter blood was spotted directly onto the Whatmann 903 cards at point-of-contact. In all surveys, cards were dried overnight and packed the next day with silica gel. Dried blood spots (DBS) were kept at room temperature and were transported to the *Laboratoire National de Sante Publique* (LNSP) in Port-au-Prince once per week where they were stored at 4°C until processed. Participants were also tested with a SD-Bioline HRP2 RDT and treated according to national guidelines if positive.

Antigen coupling to beads

Antigens were covalently coupled to unique bead regions as previously described by Rogier et al. [19]. Most of the antigen-bead sets were coupled in one batch at the start of the surveys to minimise batch-to-batch variation of antigens and bead set couplings. In addition to the malarial antigen panel, glutathione S-transferase (GST) protein was included to correct for background reactivity as participants that show high (specific or non-specific) GST responses may react to the GST-tag of malarial antigens following protein production. TT was included to act as an internal positive control as vaccinated participants as well as the negative UK control pool (see below) should show responses to this target. Antigen characteristics and details on antigen-bead coupling are depicted in Table 1.

Assay standards

Hyperimmune positive control pools

A Haitian positive serum control pool (HP) was created using country-wide, routinely collected samples from RDT positive individuals. Blood spots from participants with high responses to a range of *Pf* antigens were combined (n=63) and eluted to a standard at dilution of 1:100. A 6-point titration standard curve of 1 in 5 dilutions starting at 1:100 was created in bulk, stored at 4°C and used on each test plate. The WHO *Pf* standard 10/198 [22] was eluted in 1 ml of dH20 (1:5 serum concentration, 100 units) and subsequently in 10 ml of buffer B (phosphate-buffered saline (PBS) containing 0.5%

BSA, 0.05% Tween 20, 0.02% sodium azide, 0.5% polyvinyl alcohol, 0.8% polyvinylpyrrolidone and 0.5% w/v E. coli extract). As with the Haitian pool, a 6-point, 1 in 5 titration standard curve starting at 1:50 was created in bulk, stored at 4°C but run on only one test plate per day.

Unexposed, negative control pool and blanks

A pool of 10 serum samples from unexposed individuals from the UK was included on all the plates in Survey 1 and the first 150 plates in Survey 2 at a final serum concentration of 1:200. This pool was not included on plates in Survey 3. In addition, two blanks (buffer B) were run on each plate.

Multiplex bead assay

All samples were processed for multiplex antibody (IgG) detection using a One-Step protocol as previously described by Rogier et al. (Rogier et al., *in prep*). Briefly, one 3 mm spot was cut from the centre of a DBS and eluted overnight in 173µl of buffer B. Samples were kept at 4°C and tested within three weeks. Bead mixture was prepared by adding 6µl per bead region (250,000 beads/antigen/plate) in 5 ml of buffer A (PBS containing 0.5% BSA, 0.05% Tween-20, 0.02% sodium azide) for each plate. Bead mixture was mixed using an electronic pipette and 50µl was added to each well of a 96-well plate (Bio-Rad, Hercules, CA). Plates were placed on handheld magnetic separators (Luminex Corp) and washed three times with wash buffer (PBS containing 0.05% Tween-20). After removing plates from the separator, 50µl of anti-human secondary mixture in buffer A (1:500 biotinylated anti-human IgG, Southern Biotech; 1:1250 biotinylated anti-human IgG$_4$, Southern Biotech; and 1:200 Streptavidin conjugated to phycoerythrin, Invitrogen, Waltham, MA) was added to each well, followed by 50µl of eluted samples resulting in a final serum concentration of 1:200. Plates were incubated on a shaker overnight at 600 rpm. The next day, plates were washed five times and 100µl of PBS was added. Plates were kept on the shaker for a minimum of 30 minutes until they were read with the MagPix with Bio-Plex Manager™ MP software with a target of 20 beads/antigen/well. Median fluorescence intensity (MFI) was recorded for each sample and corrected for background reactivity by subtracting blank (buffer B only) responses by antigen (MFI corrected for background, hereafter: MFI). Results were exported to Excel workbooks per plate.

In addition, 804 samples from survey 1 were run using a Standard (hereafter: Stepwise) protocol in which, after the bead mixture wash, beads are incubated with sample for 1.5 hours (final serum

concentration of 1:100), secondary for 1 hour and buffer A for 0.5 hour (Rogier et al. 2018, *in prep*). Three washes were done in between each assay step. After the final wash, 100µl of PBS was added and plates were read as in the One-Step protocol.

Statistical analyses

Quality control

All statistical analyses were performed in R Studio version 3.3.3 [27]. Participants with GST MFI levels above the arbitrary threshold of 1000 were excluded from further analyses. All MFI values were log transformed (base 10) with MFI values smaller than background responses replaced with the mean background values for all antigens (i.e. MFI of 8.45, standard deviation: 2.36). The value of the third point of the standard HP curve of each plate was plotted in Levey-Jenning charts. Plates that fell outside of the mean +/- 2 standard deviations (SD) for two out of three highly immunogenic antigens (GLURP-R2, AMA-1 and MSP1-19) were repeated [16]. Logistic regression curves were fitted to standard curve values per plate using the *nplr* package in R Studio [28]. This function compares 2 to 5 parameter logistic regression fits and selects the fit with the smallest sum of squared errors. Logistic regression was only fitted if no more than one value of the standard curve was missing and at least one of the recorded MFI values was > 4.61 (i.e. MFI 100 before log-transformation). MFI values were first converted to proportions using the minimum and maximum MFI values for all standard curves across all antigens (2.07 and 11.17 respectively). The 5-parameter logistic regression is given below:

$$y = B + \frac{T - B}{[1 + 10^{(b*(xmid - x))}]^s}$$

B and T are the bottom and top asymptotes, b and $xmid$ are the Hill slope and the x-coordinate at the inflexion point and *s* is an asymmetry coefficient. In 4-parameter logistic regression, the s parameter is forced to be 1, while 3- or 2-parameter logistic regression force B and T to be 0 and 1, respectively. Curve parameters were recorded for each plate as well as a sequence of 200 predicted MFI values across standard curve concentrations to represent the fitted curves.

Transformation of antibody responses between the One-Step and Stepwise protocol

The sigmoidal relationship between paired log-transformed MFI measurements from 804 samples on the One-Step and Stepwise MBA protocols (Rogier et al. 2018, *in prep*) was also fitted using the *nplr* package. The fit from these models was then validated by transforming data collected on the One-

Step protocol to Step-wise responses and investigating transformed responses compared to Step-wise responses. Hereafter, recorded antibody response data from all survey samples were transformed for each antigen using these fits. Samples that fell below the bottom asymptote or over the upper asymptote, were replaced by the lowest and highest values that the model could estimate.

Results

High throughput

Nearly all collected survey samples were processed at the laboratory with minor differences due to data management issues or loss of DBS between field collection and laboratory assessment: 99.2% (5956/6006) for Survey 1; 99.6% (21,801 /21,891) for Survey 2; and 99.3% (5001/5034) for Survey 3 (Table 2). These samples were processed in 71 plates over five weeks for Survey 1; 257 plates over nine weeks for Survey 2; and 59 plates over four weeks for Survey 3. Together these represent 32,758 participant samples in eighteen weeks. After removal of high responses to GST for participants with a GST reading available (i.e. loss due to well-specific errors such as low bead counts) 5,898 samples were available in Survey 1 (99.0% of those received at the laboratory); 21,234 samples in Survey 2 (97.4%); and 4,967 samples in Survey 3 (99.3%). IgG antibody responses were successfully collected for all these participants across 21 antigens (17 *P. falciparum* antigens, 2 non-*P. falciparum* antigens and 2 non-malarial antigens), apart from minor loss of observations due to well-specific errors: 455 (0.07%), which resulted in 673,624 unique observations (Table 2).

Robust responses in both positive control standards

The highest concentrations of both the HP and the 10/198 positive control standard curves showed robust IgG responses for nearly all of the included *Pf* antigens (Figure 1). Generally higher median responses were seen in the 10/198 standard, except for LSA-1 and SBP-1, presumably due in part to the higher serum concentration. Minimal responses were recorded to HRP-2 and Hyp2 in both standards: median values in the top of the curve across all plates were similar to those of the unexposed negative control pool (median MFI < 500 before log-transformation; see Supplementary Figure 1).

Inter-plate variability

Levey-Jenning plots of IgG responses of the third point of the HP standard curve are shown in Figure 2. Plates that fell outside of the 2 SD range of mean responses for two out of three highly immunogenic antigens (GLURP-R2, AMA-1 and MSP1-19) were selected to be repeated: 2 in Survey 1, 9 in Survey 2 and 2 in Survey 3. The 5-parameter logistic regression was the optimal fit for the majority of plates (≥88% for all antigens; Supplementary Table 1) and was used for all standard curves. HP standard curves per survey are shown in Figure 3 for all antigens except HRP2 and Hyp2 (see Supplementary Figure 2 for the 10/198 curves). Inspection of the median and IQR of the y-inflection point was used to assess within and between survey variation in standard curves (Figure 4). The median and length of the IQR of y-inflection points was similar for Survey 1 and Survey 2 for most antigens; except for a smaller recorded Survey 2 median for MSP 2 CH150/9 (i.e. below the 25th percentile of Survey 1) as well as a larger Survey 2 IQR for MSP1-19, MSP 2 CH150/9, SBP1 and to a lesser extent MSP 2 Dd2. The length of the IQR for y-inflection points was generally highest in Survey 3. While for most antigens the median Survey 3 y-inflection point was similar to Survey 1 and 2, a smaller Survey 3 median (i.e. below the 25th percentile of Survey 2) was recorded for MSP 2 CH150/9, MSP 2 Dd2, GexP and borderline for SBP1. Standard curves for *Pf*, *P. vivax* (*Pv*) and *P. malariae* (*Pm*) MSP1-19 in the HP and 10/198 standard are shown in Supplementary Figure 3.

Transformation of responses between One-Step and Stepwise protocols

IgG responses collected on the One-Step protocol were transformed using to the sigmoidal relationship between paired measurements on the One-Step and the Stepwise protocol (shown in Figure 5 for AMA-1, remaining antigenic targets in Appendix B). The goodness-of-fit (GOF) ranged from 0.49 for Dd2 to 0.95 for AMA-1, while 15/17 antigenic targets had a GOF >0.70. Pearson's correlation coefficients for transformed responses compared to Stepwise responses ranged from 0.68 for MSP 2 Dd2 to 0.95 for AMA-1 and MSP1-19, while there was a strong correlation for 14/17 antigens (i.e. >0.80). Fit parameters, GOF and Pearson's correlation coefficients are summarised per antigen in Supplementary Table 2. MFI results for all survey samples were transformed using these fits.

Discussion

In this study, we described the in-country application and retrospective quality control of an MBA simultaneously detecting IgG responses to seventeen *P. falciparum* recombinant antigens and peptides in Haiti. Antibody measurements were collected for 32,758 participant samples across three surveys in eighteen weeks. Results for only 0.2-0.5% of the participants per survey had to be removed

due to high GST responses (i.e. MFI measurements corrected for background >1000). The data collected for the remaining participants, represent 545,683 *P. falciparum* serological data points of which only 414 (0.08%) had to be removed due to well-specific errors such as low bead counts. The quality control to assess the precision of the assay was based on a specifically created positive control standard of hyperimmune sera from RDT positive Haitians (Haitian hyperimmune sera pool: HP). In addition, we included the WHO *Pf* 10/198 reference standard on one plate per day to compare results where needed [22]. The MBA was implemented as a high-throughput tool enabling rapid turnaround of antibody measurements for epidemiological surveys which aimed to directly inform control and elimination policies.

Inter-plate variability was assessed using Levey-Jenning plots which showed no trends in loss or gain of IgG responses in the HP standard over time during assay processing other than minimal daily fluctuations. Daily fluctuations could have been caused by inter-technician variability, pipetting errors or fluctuations in laboratory temperatures and/or incubation time during assay processing. Plates that fell outside the mean +/- 2 SD of responses in the third point of the HP standard curve (i.e. the third point in the six-point dilution series of the HP standard) for two out of three highly immunogenic targets were repeated as previously described by others [16]. Using multiple targets for this selection compared to one target avoids rejecting a plate due to well-specific errors such as low bead counts or pipetting errors. Inter-plate variability was further assessed using 5-parameter logistic regression for standard curves on each plate [25,29]. Inspection of the median and IQR of the y-inflection point was used to assess within and between survey variation in standard curves. The median and length of the IQR of y-inflection points was similar for Survey 1 and Survey 2 for most antigens. The length of the IQR for y-inflection points was generally highest in Survey 3. For four of the included targets, the standard curves revealed a loss of reactivity over time (MSP2 Dd2, MSP 2 CH150/9, GexP and SBP1). As beads were coupled in one batch at the start of the first survey to exclude variations between bead batches, the loss in reactivity may be explained by these antigens being less stable after long-term storage. Therefore, survey results for these targets should be interpreted with caution and future use of these antigens would need to optimise storage and binding conditions.

The application of the WHO *Pf* 10/198 reference standard to the MBA was recently described by Ubillos et al. [15]. They showed robust IgG responses to twenty-three antigens, twenty of which were malarial antigenic targets. Here, we reported antibody responses in this reference standard to fourteen novel recombinant malarial antigens of which twelve showed robust responses (Hyp-2 and

HRP2 did not). Responses to *Pf*, *Pv* and *Pm* MSP1-19 were similar to those described when the reference standard was developed and tested on ELISA [22]. By adding this WHO *Pf* 10/198 reference standard to one plate per day alongside the newly developed hyperimmune pool of Haitian sera on every plate, we were able to confirm the presence or absence of trends over time in antigen-specific results. However, standard curves from this pool were more variable between plates and surveys which could partly be due to the smaller sample size and/or this reagent may be more sensitive to variation in incubation times during assay processing or long-term storage.

The One-Step assay protocol used in this study was recently described by Rogier et al. (Rogier et al. 2018, *in prep*). Sero-prevalence estimates using this One-Step protocol were similar to those recorded by the conventional (Step-wise) protocol. The One-Step protocol allows rapid data collection and increases the ease-of-use of the assay. Nevertheless, the saturation seen at the higher range of MFI levels may limit the ability to detect a decrease in antibody levels after the implementation of interventions if the surveys are close together in time. Moreover, it would limit direct comparison of results with studies that collected data on a conventional protocol. Here, we used the sigmoidal relationship from paired log-transformed MFI measurements on each protocol to transform the survey data collected on the One-Step protocol to Step-wise/conventional responses. Highly immunogenic antigens (i.e. those eliciting high antibody titres), such as AMA-1, MSP1-19 and GLURP-R2, generally generated a better fit than less immunogenic targets, such as H103 and Hyp2, as saturation was more pronounced thus creating a full sigmoidal curve. Although it should be noted that the comparison data was run during a specific window of time, this approach allowed for re-creation of Stepwise/conventional MFI measurements as shown by the strong correlation between transformed and Stepwise/conventional responses (i.e. Pearson's correlation coefficient >0.8 for 14/17 antigens).

Conclusion

In this study, we have described the successful in-country application of the MBA with highly efficient throughput and acceptable inter-plate variability for well-characterised antigenic targets in Haiti. This assay allows for rapid assessment of the exposure history of populations which can directly inform control and elimination policies. However, inter-plate variability was considerable for newly described targets with lower immunogenicity which are perhaps more sensitive to long-term storage, fluctuations in laboratory temperatures and/or incubation time during assay processing. Future work

should focus on further evaluation of the WHO *Pf* 10/198 in assessing stability of repeated measurements in serial dilutions over time as well as the effects of long-term storage conditions.

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Ethics approval and consent to participate

The LSHTM Research Ethics Committee (10393), Tulane Institutional Review Board (794709) and the National Bioethics Committee in Haiti (1516-30) approved survey 1 and Survey 3. The CDC Institutional Review Board (6821), LSHTM Research Ethics Committee (10466) and the National Bioethics Committee in Haiti (1516-29 and 1617-31) approved Survey 2. All participants provided informed written consent or assent. The anonymised malaria-naïve serum samples were collected by Public Health England (LSHTM Research Ethics Committee: 11684).

Authors' contributions

JFL, MC, TPE, TD, RA, GS, CD and ER designed the surveys. TPE, TD, RA, VJ, GS, KH and MC supported/performed field data collection. JP, IR, TE and GM performed laboratory data collection. LLvdH, AE, JB, KKAT, CD and ER supported laboratory data collection. LLvdH, KKAT, CD and ER analysed and interpreted the data. LLvdH wrote the first draft of the manuscript with support from ER and CD. All authors read and approved the final manuscript.

Table 1: Characteristics of multiplex bead assay antigen panel for three malaria transmission surveys in Haiti. iRBC: infected red blood cell. PVM: parasitophorous vacuole membrane. *Helb et al. [4].

Order	Antigen	Alias	Pathogen/Vector	Description	Location	Expression tag	Strain	Rationale	Antigen ug/mL beads	Coupling pH	Reference
1	Etramp 4 Ag 2	etr42	*P. falciparum*	Early transcribed membrane antigen. Integral PVM protein. C-terminal	iRBC, PVM	GST	3D7	Recent malaria exposure*	115	7.2	Helb et al. (PMID: 26216993); Tetteh unpublished
2	Etramp 5 Ag 1	etr51	*P. falciparum*	Early transcribed membrane antigen. Integral PVM protein.	iRBC, PVM	GST	3D7	Recent malaria exposure*	100	7.2	Spielmann et al. (PMID: 12686607); Tetteh unpublished
3	GexP	gexp	*P. falciparum*	Gametocyte exported protein 18. Unknown function.	iRBC/ Gametocyte	GST	3D7	Recent malaria exposure*	200	7.2	Helb et al. (PMID: 26216993); Tetteh unpublished
4	H103	h103	*P. falciparum*	H103/merozoite surface protein 11	Merozoite surface/rophtry neck	GST	3D7	Malaria exposure	100	7.2	Pearce et al. (PMID: 15664649)
5	HRP2	hrp2	*P. falciparum*	Histidine rich protein 2	iRBC and secreted	GST	Type A and B	Malaria exposure	25	5	Rogier et al. (PMID: 28192523)
6	HSP40 Ag1	hsp40	*P. falciparum*	Heat shock protein 40	iRBC	GST	3D8	Recent malaria exposure*	100	7.2	Helb et al. (PMID: 26216993); Tetteh unpublished
7	Hyp 2	hyp2	*P. falciparum*	Plasmodium exported protein	Hypothesised location: iRBC	GST	3D7	Recent malaria exposure*	1000	7.2	Helb et al. (PMID: 26216993); Tetteh unpublished
8	LSA-1	lsa1	*P. falciparum*	Liver surface antigen 1	Infected hepatocyte	N/A	Synthesized peptide, Pl1043 epitope	Malaria exposure (liver stage)	60	5	Plucinski et al. (PMID: 29444078)
9	MSP2 CH150/9	msp2_ch150	*P. falciparum*	CH150/9 allele of MSP2. Full-length.	Merozoite surface	GST	CH150/9	Malaria exposure	5	5	Roy (PMID: 16111789)
10	MSP2 Dd2	msp2_dd2	*P. falciparum*	Dd2 allele of MSP2. Full-length.	Merozoite surface	GST	Dd2	Malaria exposure	20	5	Taylor et al. (PMID: 7591074)
11	PfAMA1	ama1	*P. falciparum*	Apical membrane antigen 1	Micronemes	His	FVO	Malaria exposure	15	7.2	Collins et al. (PMID: 17192270)
12	PfGLURP R0	glurp0	*P. falciparum*	Glutamate rich protein R0	Merozoite surface	N/A	Synthesized peptide, R0 fragment	Malaria exposure	30	5	Kerkhof et al. (PMID: 27809852)
13	PfGLURP R2	glurp2	*P. falciparum*	Glutamate rich protein R2	Merozoite surface	His_{x6}	F32	Malaria exposure	15	7.2	Theisen et al. (PMID: 7719909)
14	PfMSP1-19	msp119	*P. falciparum*	19kDa fragment of MSP1 molecule	Merozoite surface	GST	Wellcome	Malaria exposure	20	7.2	Burghaus and Holder (PMID: 8078519)
15	PfSEA	sea	*P. falciparum*	Schizont egress antigen	iRBC	GST	3D7	Malaria exposure	20	5	Raj et al. (PMID: 24855263); Tetteh unpublished
16	PmMSP1-19	pmmsp119	*P. vivax*	19kDa fragment of MSP1 molecule	Merozoite surface	GST	Pm China I	Pv Malaria exposure	20	5	Priest et al. (PMID: 30413163)
17	PvMSP1-19	pvmsp119	*P. malariae*	19kDa fragment of MSP1 molecule	Merozoite surface	GST	Pv Belem	Pm Malaria exposure	20	5	Priest et al. (PMID: 30413163)
18	rCSP	rcsp	*P. falciparum*	Circumsporozoite surface protein	Sporozoite	N/A	3D7	Recent malaria exposure* (sporozoite stage)	60	7.2	Kastenmuller et al. (PMID: 23275094)
19	SBP1	sbp1	*P. falciparum*	Skeleton-binding protein; Maurer's cleft.	iRBC	GST	3D7	Malaria exposure	15	5	Gruring et al. (PMID: 21266965); Tetteh unpublished
21	GST	gst	*S. japonicum*	Glutathione S-transferase				Correct for background reactivity due to GST-tag	20	5	J. Priest/CDC
22	Tetanus Toxoid	tt	*C. tetani*	Tetanus Toxoid				Vaccination target: internal "positive" control	12.5	5	Massachusetts Biologic Laboratories

Table 2: Number of samples and observations for which Immunoglobulin G (IgG) antibody responses were successfully collected using a multiplex bead assay across three malaria transmission surveys in Haiti.

	Survey 1	Survey 2	Survey 3
Plates, n	71	257	59
Samples, n Proportion of previous n			
- Collected in the field	6006	21891	5034
- Received/processed at the lab	5956 99.17%	21801 99.59%	5001 99.34%
- GST reading available	5922 99.43%	21336 97.87%	4989 99.76%
- Acceptable GST reactivity	5898 99.59%	21234 99.52%	4967 99.56%
Observations*, n Loss, n			
- All antigens (n=21) Loss	123,850 8	445,787 127	103,987 320
- *Plasmodium* antigens (n=19) Loss	112,054 8	403,325 121	94,059 314
- *P. falciparum* antigens (n=17) Loss	100,260 6	360,872 106	84,137 302

*Unique IgG observations successfully collected (i.e. number of participants multiplied by number of antigens/peptides to which antibody responses were collected).
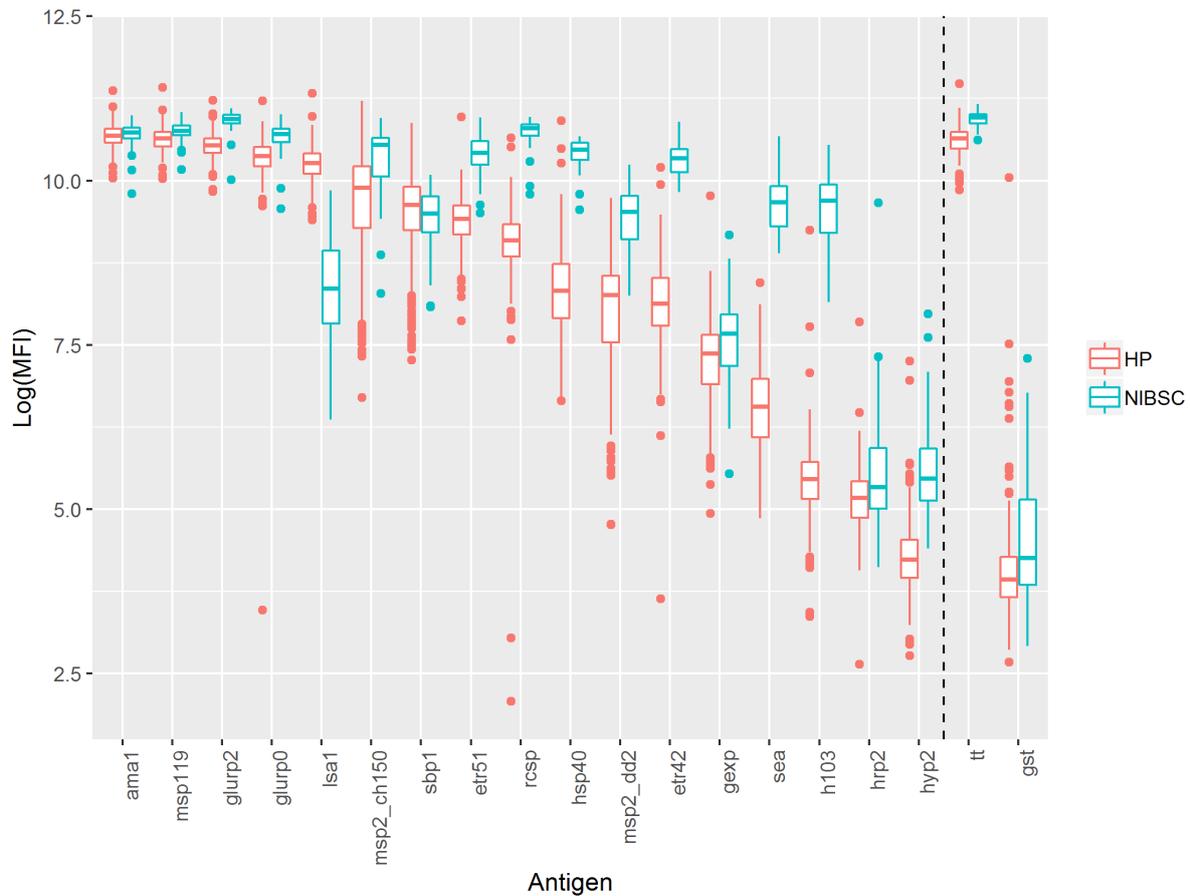
Figure 1: Antibody reactivity profile of standards of hyperimmune sera. MFI: Median fluorescence intensity values were corrected for background reactivity of blank responses and log-transformed (y-axis). For antigen (x-axis) acronyms see Table 1. In addition to malarial antigens, tetanus toxoid (tt) and glutathione S-transferase (gst) responses are shown. HP: Haitian hyperimmune sera pool (for details see main text). NIBSC: *Plasmodium falciparum* 10/198 WHO standard [22]. The HP curve was run on every plate, while the 10/198 curve was run on one plate per day. Responses to the first point of the curve are shown, with a serum concentration of 1:200 for the HP and 1:100 for the 10/198 standard. Antigens are ordered by median HP responses.
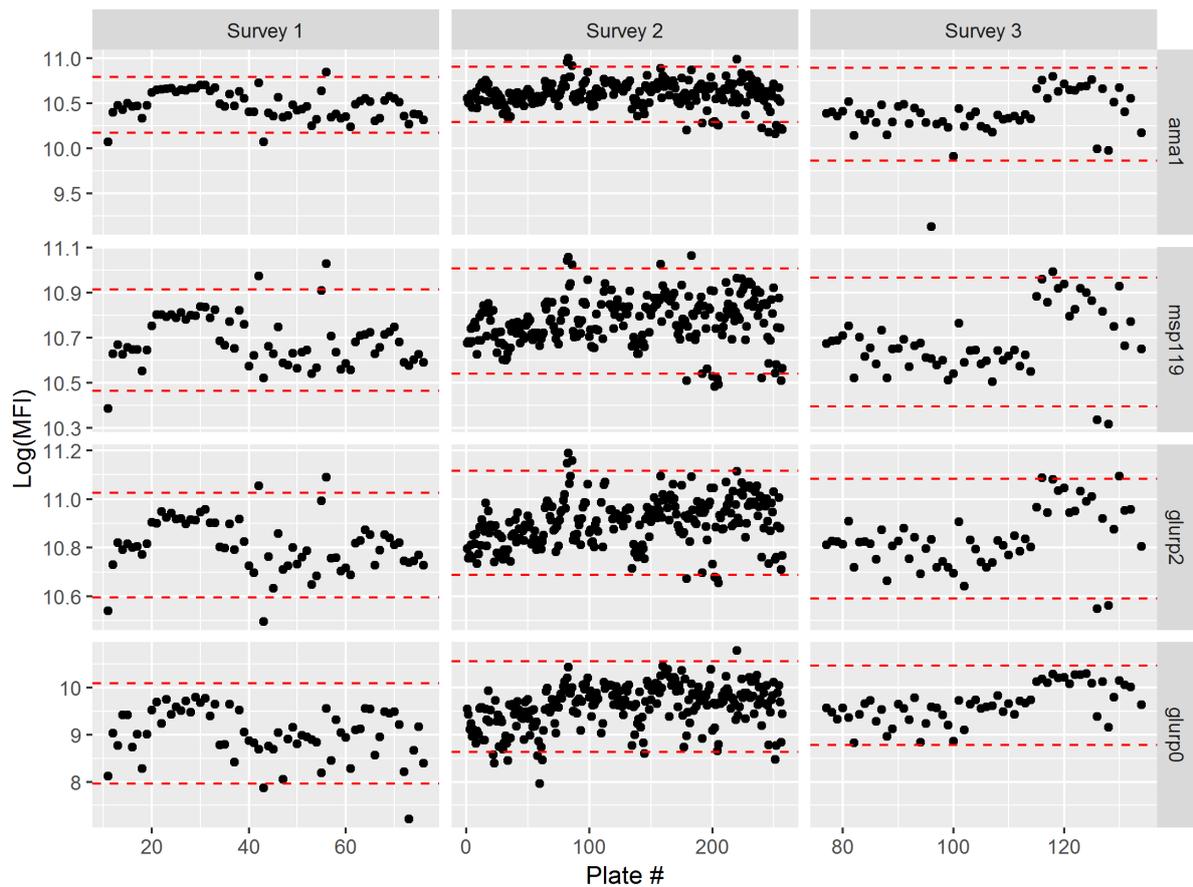
Figure 2: Levey-Jenning charts of antibody responses in the standard of Haitian hyperimmune sera across all plates. MFI: Median fluorescence intensity; values were corrected for background reactivity of blank responses and log-transformed. HP: Haitian hyperimmune sera pool (for details see main text). Responses in the third point of the curve (serum concentration of 1:5,000) are shown across three surveys. The mean plus/minus two times the standard deviation across responses per survey are shown in dashed red lines.
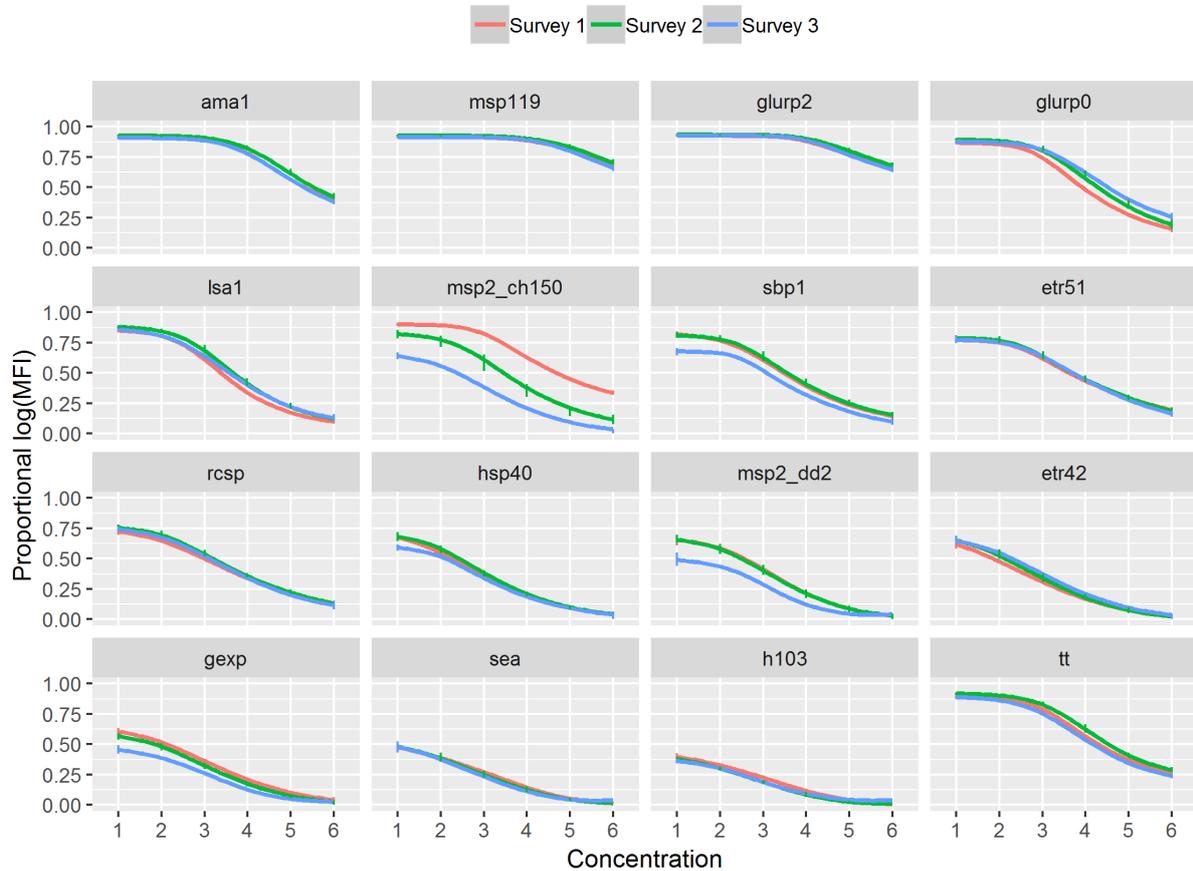
Figure 3: Average standard curves of the standard of Haitian hyperimmune sera for each survey.
MFI: Median fluorescence intensity. For each plate and antigen, HP standard curves were fitted using 5-parameter logistic regression. Standard curves were only fitted if the non-log-transformed MFI of at least one of the dilution points was larger than 100. Using the curve parameters, MFI values were predicted across a sequence of 200 values of standard curve concentrations for each of the plates. Standard curves per survey were plotted using the generalized additive model method and the interquartile range is shown in vertical lines at each of the dilution steps of the standard curve.

Figure 4: Median and interquartile range of predicted y-inflection points of standard curves per survey using the Haitian standard of hyperimmune sera. Median and interquartile range of the predicted y-inflection points from standard curves for all plates using 5-parameter logistic regression. For antigen (x-axis) acronyms see Table 1. Antigens are ordered by median responses as shown in Figure 1.
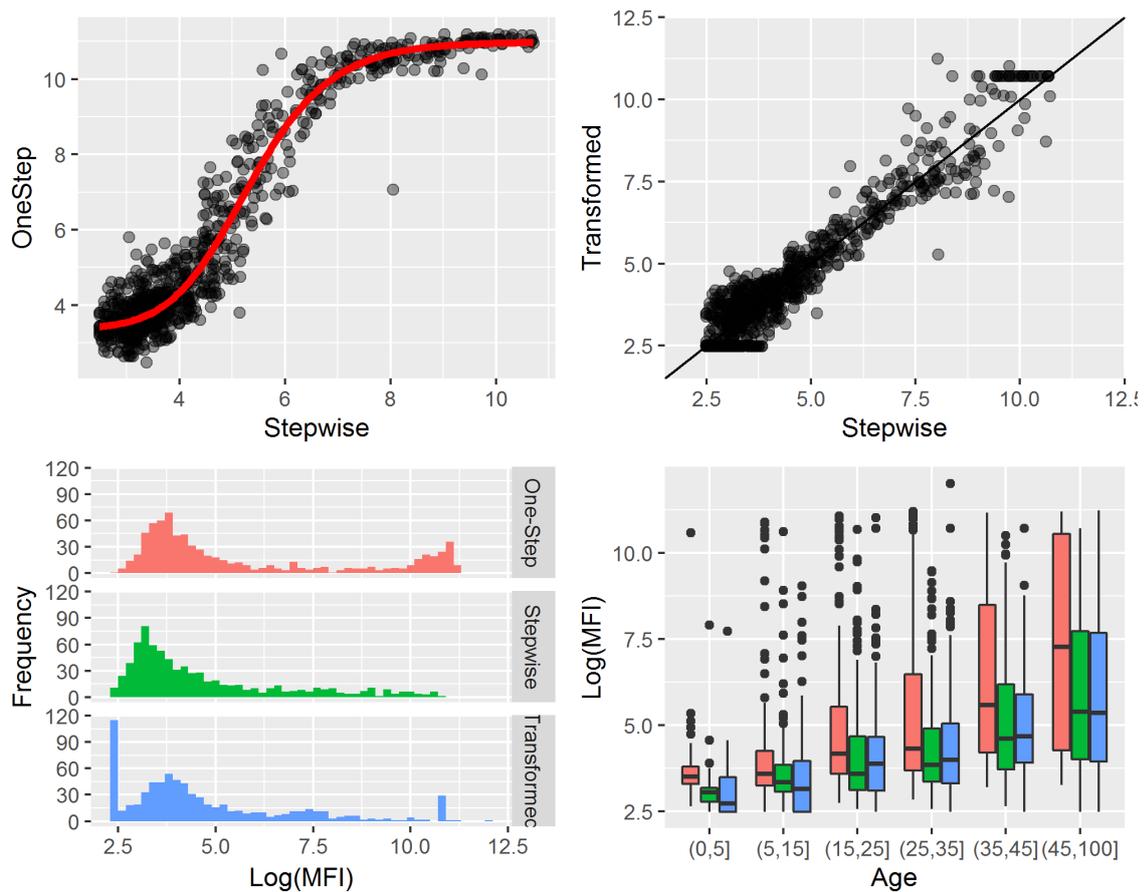
Figure 5: Transformation of antibody responses between the OneStep and Stepwise multiplex bead assay protocols for AMA-1. MFI: Median fluorescence intensity. The sigmoidal relationship of paired measurements was obtained using 804 samples processed on the One-Step and the Stepwise/conventional assay protocol (Rogier et al., *in prep*). Recorded responses lower than blank responses were replaced with the average of blank MFI values of the One-Step protocol across all plates by antigen. Measurements from the One-Step protocol were transformed using the sigmoidal fit. Samples that fell below the bottom asymptote or over the upper asymptote, were replaced by the lowest and highest values that the model could estimate. Plots for remaining antigenic targets are in Appendix B and curve parameters are in Supplementary Table 2. (a) Scatter plot of paired measurements with 5-parameter logistic regression in red line. (b) Scatter of transformed responses compared to measurements using the Stepwise/conventional protocol. (c) Histograms of One-Step, Stepwise/conventional and transformed antibody measurements. (d) Box plots of One-Step, Stepwise/conventional and transformed antibody measurements over age groups.

Supplementary Tables

Supplementary Table 1: Frequency of plates with n-parameter logistic regression fit for standard curves per antigen across three surveys in Haiti. For antigen abbreviations, see **Chapter 5**, Table 1. HP: standard of Haitian hyperimmune sera (for details see main text). 10/198: *Plasmodium falciparum* 10/198 WHO hyperimmune standard [22]. The total number of plates is variable per antigen as logistic regression fits for standard curves were only applied if the non-log-transformed MFI of at least one of the dilution points was larger than 100.

Antigen	Standard	n-parameter		
		3	4	5
msp2_ch150	HP	1	0	354
	10/198	0	7	42
msp2_dd2	HP	1	4	351
	10/198	0	15	35
etr42	HP	0	14	342
	10/198	0	6	44
etr51	HP	0	0	357
	10/198	0	2	48
Gexp	HP	0	6	350
	10/198	0	16	34
glurp0	HP	0	1	356
	10/198	0	12	38
glurp2	HP	0	10	347
	10/198	5	17	28
h103	HP	1	14	198
	10/198	0	4	46
hsp40	HP	0	10	347
	10/198	0	2	48
ama1	HP	0	42	315
	10/198	0	20	30
msp119	HP	6	24	326
	10/198	2	23	25
lsa1	HP	0	0	357
	10/198	2	16	32
rcsp	HP	0	4	353
	10/198	0	6	44
sbp1	HP	0	3	354
	10/198	0	6	45
sea	HP	1	13	325
	10/198	0	3	47
tt	HP	0	3	354
	10/198	2	30	18
pmmsp	HP	0	79	276
	10/198	2	12	36
pvmsp	HP	0	8	58
	10/198	0	5	23

Supplementary Table 2: Curve parameters of logistic regression fits for antibody responses recorded by the OneStep and Stepwise multiplex bead assay protocol for each antigen. Median fluorescence intensity (MFI) measurements were corrected for background reactivity of blank responses and natural log-transformed. The sigmoidal relationship of paired median fluorescence intensity (MFI) measurements was obtained using 804 samples processed on the One-Step and the Stepwise assay protocol (Rogier et al., *in preparation*, **Appendix A**). Curve parameters from sigmoidal logistic regression fits include the number of parameters (**npar**), the top asymptote (**max**), the mid-point (**mid**), the slope, the bottom asymptote (**min**), the asymmetry parameter (**s**) and the goodness-of-fit estimates (**GOF**). Recorded MFI measurements lower than blank responses were replaced with the average of blank MFI values of the One-Step protocol across all plates by antigen. Measurements from the One-Step protocol were transformed using the sigmoidal fit. Samples that fell below the bottom asymptote or over the upper asymptote, were replaced by the lowest and highest values that the model could estimate. The Pearson correlation coefficients for transformed responses compared to Stepwise responses is also included this table (**Pearson**).

Antigen	npar	max	mid	slope	min	s	gof	Pearson
msp2_ch150	5	11.136	6.470	0.485	3.462	0.903	0.829	0.868
ama1	5	10.988	4.542	0.485	3.362	1.978	0.946	0.954
sbp1	5	11.768	5.076	0.216	1.812	1.206	0.739	0.867
msp119	5	11.283	1.187	0.294	3.689	18.427	0.920	0.949
hyp2	5	7.567	3.295	0.401	3.832	4.138	0.736	0.855
gexp	4	24.366	6.528	0.056	-9.926	1.000	0.794	0.891
lsa1	4	10.951	5.835	0.455	3.380	1.000	0.764	0.783
hrp2	5	10.851	5.110	0.489	3.858	1.990	0.803	0.823
glurp0	5	10.847	8.836	1.313	3.221	0.145	0.843	0.802
etr51	5	10.697	7.489	0.561	2.970	0.399	0.774	0.804
hsp40	5	10.262	6.332	3.942	3.427	0.095	0.846	0.835
rcsp	5	11.126	7.274	0.571	2.957	0.402	0.839	0.874
h103	4	11.203	6.679	0.380	3.526	1.000	0.671	0.716
glurp2	5	11.154	8.213	0.645	3.309	0.489	0.913	0.900
etr42	5	10.074	7.199	2.242	3.182	0.105	0.791	0.840
Sea	5	11.340	6.290	0.379	3.967	1.504	0.837	0.839
msp2_dd2	4	9.998	5.982	0.395	3.195	1.000	0.488	0.684

Supplementary Figures
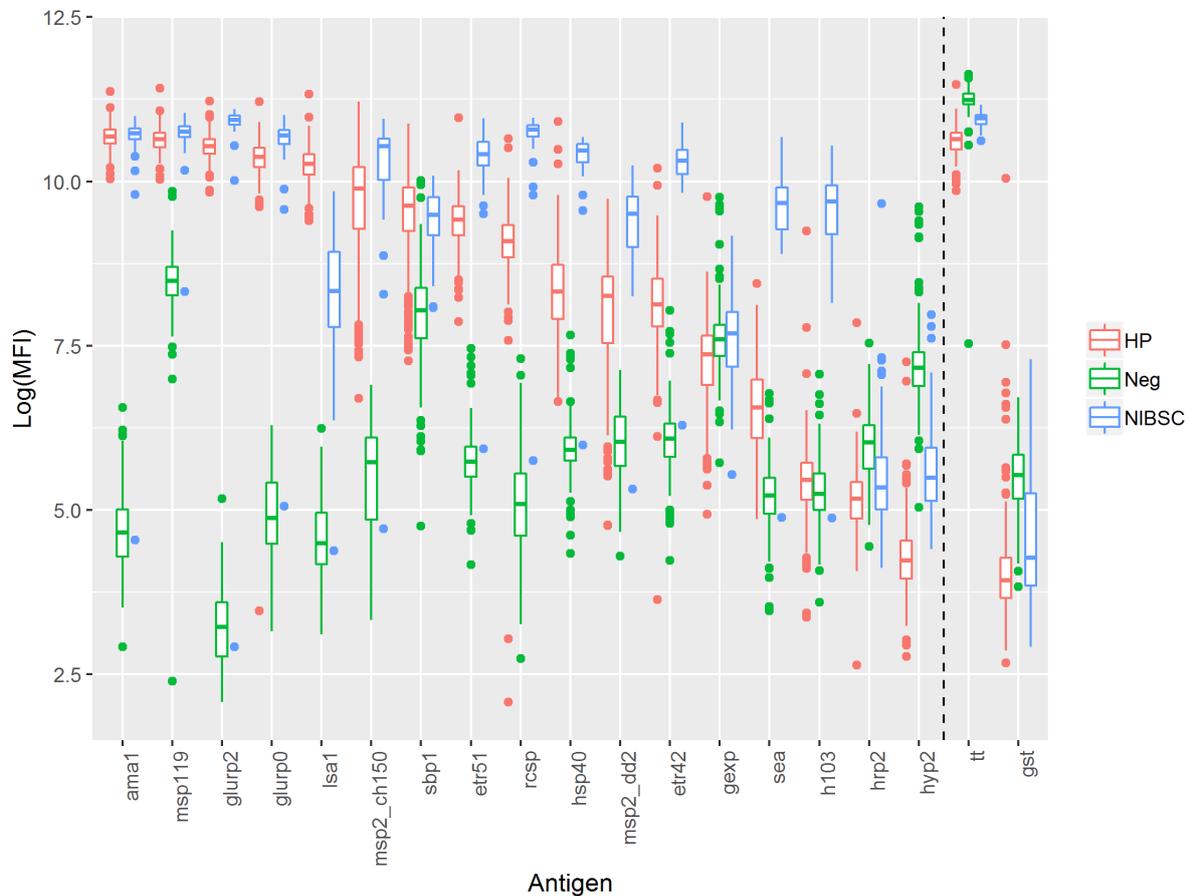
Supplementary Figure 1: Antibody reactivity profile of positive control standards of hyperimmune sera as well as a and negative control standard of malaria-unexposed sera. Median fluorescence intensity (MFI) measurements were corrected for background reactivity of blank responses and natural log-transformed (y-axis). For antigen (x-axis) abbreviations see **Chapter 5**, Table 1. In addition to malarial antigens, tetanus toxoid (tt) and glutathione S-transferase (gst) responses are shown. HP: standard of Haitian hyperimmune sera (for details see main text). NIBSC: *Plasmodium falciparum* 10/198 WHO hyperimmune standard [22]. Neg: pool of 10 serum samples from malaria unexposed individuals from the UK. The HP curve was run on every plate, while the 10/198 curve was run on one plate per day. Responses to the first point of the curve are shown, with a serum concentration of 1:200 for the HP and Neg, and 1:100 for the 10/198 standard. Antigens are ordered by median HP responses.
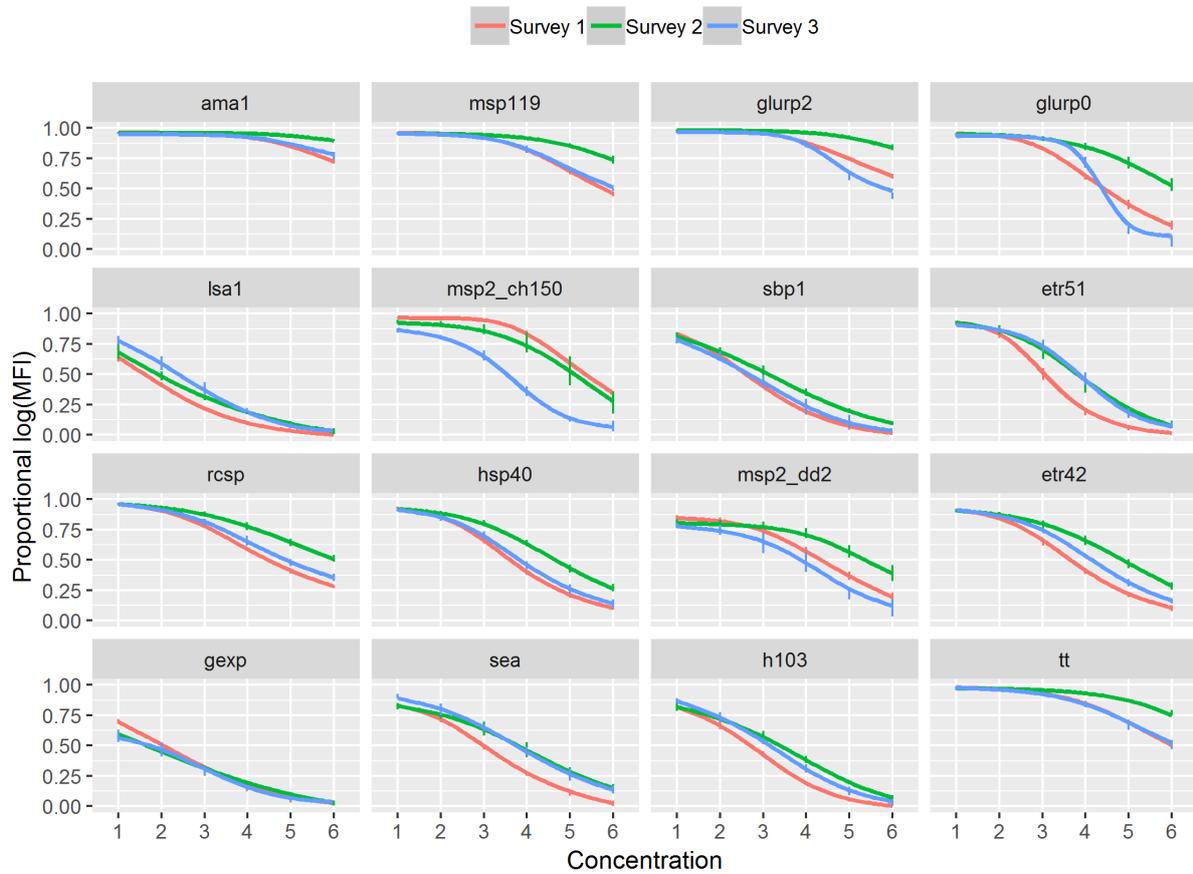
Supplementary Figure 2: Average standard curves of the 10/198 WHO positive control standard of hyperimmune sera for each survey. Median fluorescence intensity (MFI) measurements were corrected for background reactivity of blank responses and natural log-transformed. MFI values were converted to proportions using the minimum and maximum MFI values for all standard curves across all antigens (2.07 and 11.17 respectively). For antigen abbreviations see **Chapter 5**, Table 1. For each plate and antigen, 10/198 WHO standard curves were fitted using 5-parameter logistic regression. Standard curves were only fitted if the non-log-transformed MFI of at least one of the dilution points was larger than 100. Using the curve parameters, MFI values were predicted across a sequence of 200 values of standard curve concentrations for each of the plates. Standard curves per survey were plotted using the generalized additive model method and the interquartile range is shown in vertical lines at each of the dilution steps of the standard curve.

Supplementary Figure 3: Average standard curves of the Haitian (HP, top) and 10/198 WHO (NIBSC, bottom) positive control standard of hyperimmune sera for each survey for the *Plasmodium falciparum*, *P. vivax* and *P. malariae* 19 kDa merozoite surface protein 1. Median fluorescence intensity (MFI) measurements were corrected for background reactivity of blank responses and natural log-transformed. MFI values were converted to proportions using the minimum and maximum MFI values for all standard curves across all antigens (2.07 and 11.17 respectively). For antigen abbreviations see **Chapter 5**, Table 1. For each plate and antigen, HP and 10/198 WHO standard curves were fitted using 5-parameter logistic regression. Standard curves were only fitted if the non-log-transformed MFI of at least one of the dilution points was larger than 100. Using the curve parameters, MFI values were predicted across a sequence of 200 values of standard curve concentrations for each of the plates. Standard curves per survey were plotted using the generalized additive model method and the interquartile range is shown in vertical lines at each of the dilution steps of the standard curve.

Additional File: Comparison of median fluorescence intensity measures in serum compared to dried blood spot eluate using OneStep and Stepwise multiplex bead assay protocols

Corran et al. have previously shown that paired samples of serum and blood spots from Tanzania showed similar recoveries of antibodies using enzyme-linked immunosorbent assay (ELISA) [1]. However, how these compare for multiplex bead assay (MBA) remains largely unknown. To compare MBA median fluorescence intensity (MFI) measures in serum and DBS eluate, samples from survey participants in Haiti were analysed.

Methods

Participant samples were collected as part of Survey 1 in Artibonite, central Haiti (**Chapter 5**). Whole-blood from finger-prick were collected in EDTA microtainers (Safe-T-Fill™ Capillary Blood Collection Systems: EDTA, # 07 7053, RAM Scientific Inc., Yonkers, NY). Three 60 µl spots were pipetted on Whatmann 903 cards (GE Healthcare) at the end of each day in a field laboratory in Artibonite. Whatmann 903 cards were dried overnight and stored at ambient temperate until transport. Remaining whole blood was kept in EDTA microtainers at +4°C in the field laboratory. DBS and EDTA microtainers were transported to the *Laboratoire National de Santé Publique* (LNSP) in Port-au-Prince once per week. At LNSP, DBS and EDTA microtainers were stored at +4°C. Serum was separated using centrifugation at 5000g for 2 minutes and aliquoted to 96-well storage plates (Axygen 500 µl round-bottom, Fisher Scientific, # 14-222-234) within 3 days after arrival. Separate 96-well storage plates were prepared diluting serum and DBS eluate 1:100 in buffer B. These were incubated overnight and stored at +4°C until sample processing.

Antimalarial IgG responses in diluted serum and DBS eluate were analysed by MBA using the OneStep and Stepwise (i.e. Standard/conventional) protocol. Details on these protocols can be found in **Chapter 5** and **Appendix A**. Serum and DBS eluate was tested at a final concentration of 1:200. MFI values were recorded on the MAGPIX with Bio-Plex Manager™ MP software and corrected for blank (buffer B) responses as described in **Chapter 5**. We aimed to test the first 800 samples that came into the lab for Survey 1 which resulted in 796 DBS samples (loss due to well-specific errors such as low bead counts), for which 712 paired serum samples were available (loss due to insufficient serum available to aliquot in the field laboratory or at LNSP). Statistical analyses were performed in R Studio Version 1.1.456.

Results

Antibodies to six antigens were selected: three relatively high immunogenicity targets (GLURP-R2, AMA-1 and Rh2-2030) and three relatively low immunogenicity targets (Etramp 5 Ag 1, HSP40 and LSA-1). Visual inspection of scatter plots comparing MFI measurements in serum and DBS eluate using the Stepwise MBA protocol reveals a strong linear correlation, though generally MFIs recorded in serum are above the line of equality (Figure 1). Some clear outliers can be seen, mostly for GLURP-R2 (n~11). For the OneStep protocol, the same pattern of increased MFI measurements in serum is observed though overall comparisons are less linear and correlated (Figure 2). MFIs in serum saturate for GLURP-R2.

Conclusion

Paired DBS and serum samples show similar recoveries of antibodies using the MBA Stepwise protocol. The minimal increase in MFI measurements for serum samples is as expected as perhaps not all antibodies elute efficiently from DBS. For the OneStep protocol, recoveries were less linear possibly due to differences in incubation times (as incubation is overnight and therefore time windows may vary) or prozone phenomenon (i.e. false-negative results resulting from high antibody titres) [2]. Outliers on either protocol may also be due to pipetting errors.

DBS are logistically more convenient than serum samples due to ease of collection, storage and transportation. Moreover, DBS are simultaneously a source of DNA for PCR-based methods to diagnose malaria infections. The fact that similar antibody recoveries were shown by MBA between these sample types, confirms previously described results for ELISA [1]. Based on these results, all participant samples in remaining project surveys were collected on Whatman 903 cards and stored as DBS.

References

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2. Schnaidt M, Weinstock C, Jurisic M, Schmid-Horch B, Ender A, Wernet D. HLA antibody specification using single-antigen beads--a technical solution for the prozone effect. Transplantation. 2011;92:510–5.
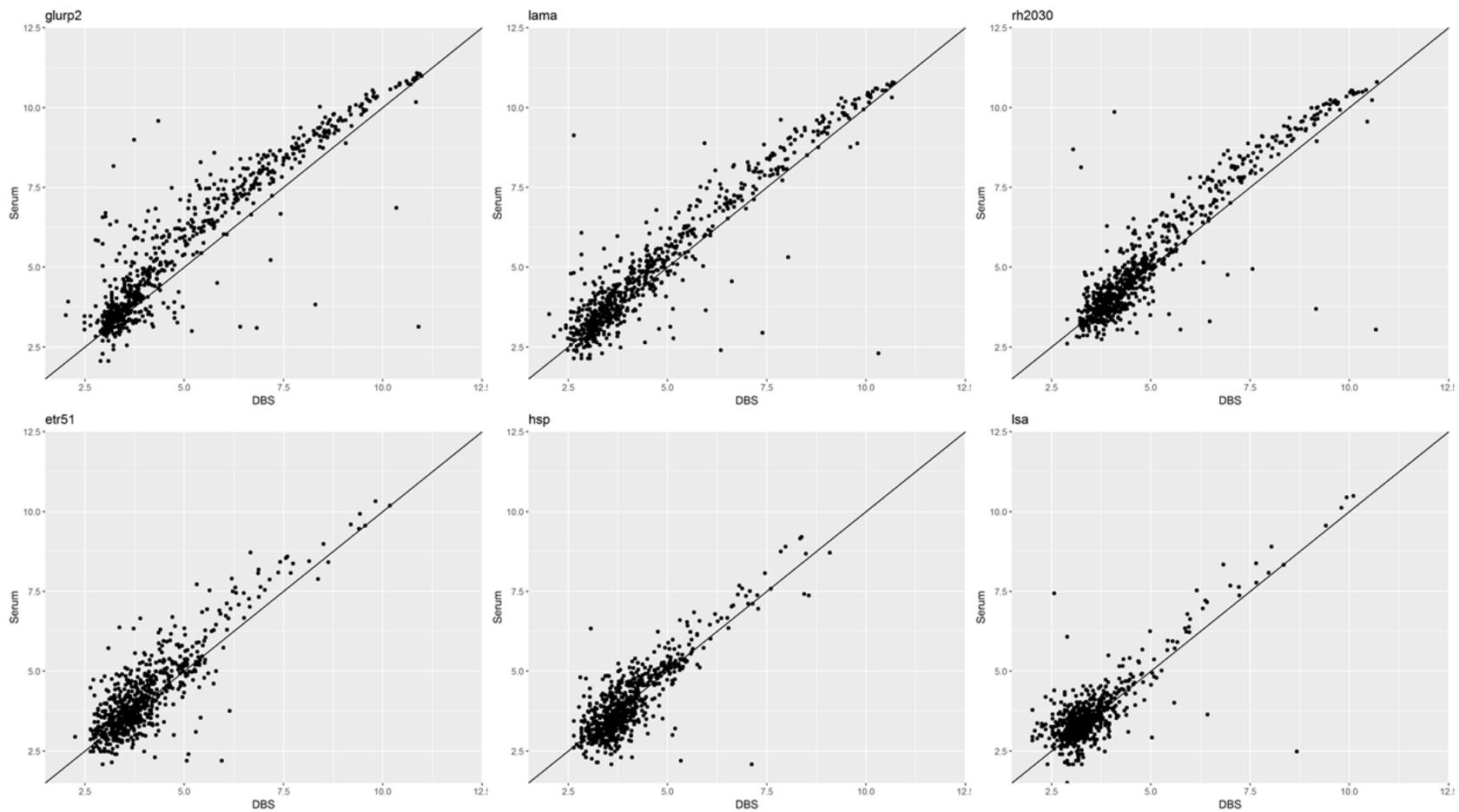
Figure 1: Comparison of median fluorescence intensity (MFI) measures in serum (y-axes) and dried blood spots (DBS) eluate (x-axes) as recorded by the multiplex bead assay Stepwise protocol. MFI values are natural log-transformed and corrected for background reactivity (i.e. blank, buffer B responses). Black dots represent 712 participant samples from Survey 1, while the line of equality is shown in black. For antigen abbreviations, see Table 1 (**Chapter 5**).
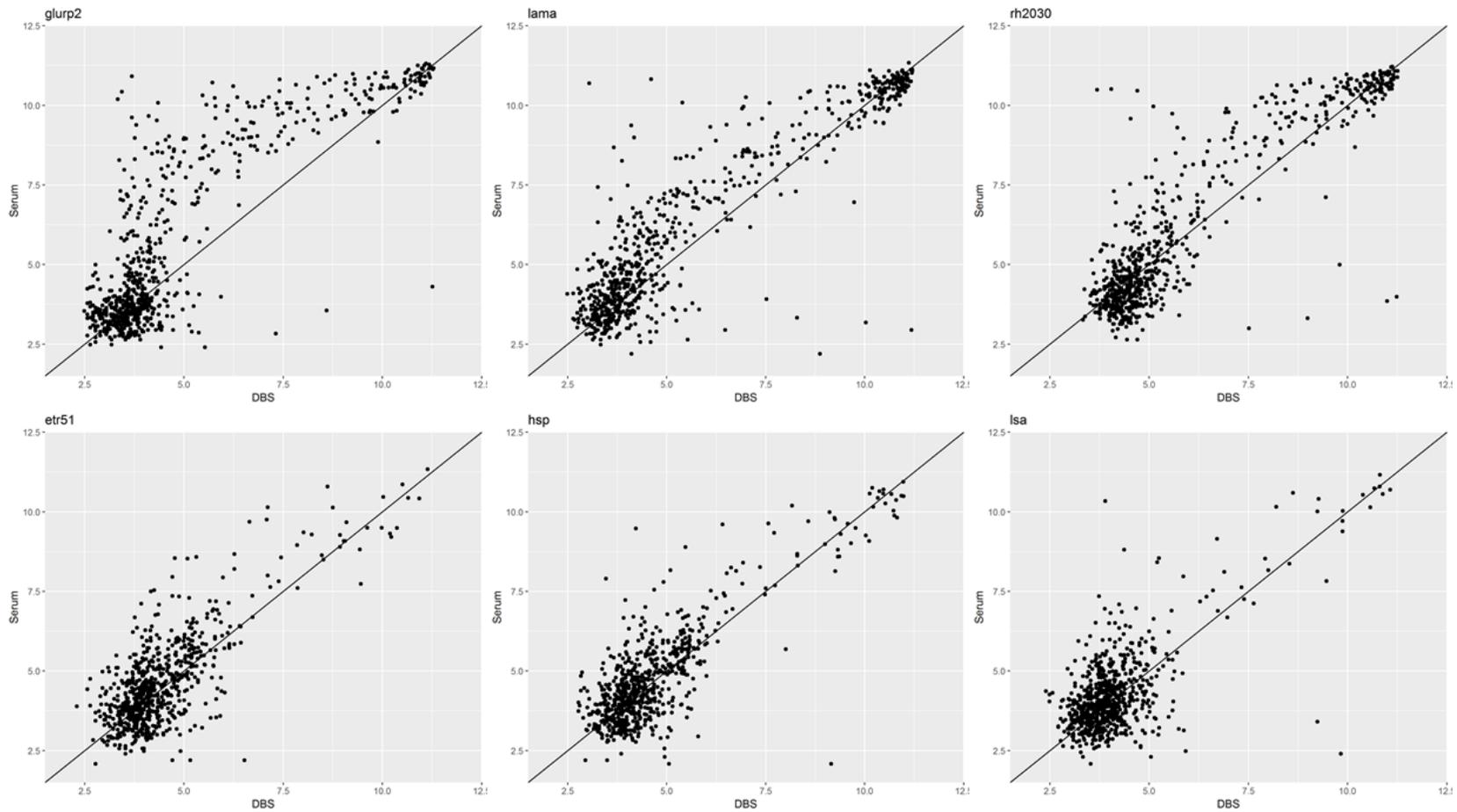
Figure 2: Comparison of median fluorescence intensity (MFI) measures in serum (y-axes) and dried blood spots (DBS) eluate (x-axes) as recorded by the multiplex bead assay OneStep protocol. MFI values are natural log-transformed and corrected for background reactivity (i.e. blank, buffer B responses). Black dots represent 712 participant samples from Survey 1, while the line of equality is shown in black. For antigen abbreviations, see Table 1 (**Chapter 5**)

Chapter 6: Antimalarial Antibody Detection Assays: In Search of a Standardised Tool to Confirm the Absence of Malaria Transmission

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SECTION A – Student Details

Student	Lotus Leonie van den Hoogen
Principal Supervisor	Chris Drakeley
Thesis Title	The use of antimalarial antibodies to measure transmission in low transmission and pre-elimination settings

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?			
When was the work published?			
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion			
Have you retained the copyright for the work?*	Choose an item.	Was the work subject to academic peer review?	Choose an item.

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SECTION C – Prepared for publication, but not yet published

| Where is the work intended to be published?	Scientific Reports
Please list the paper's authors in the intended authorship order:	Lotus L. van den Hoogen, Nuno Sepúlveda, Paolo Bareng, José Moniz Fernandes, Ralph Reyes, Joana Alves, Júlio Rodrigues, Katie Patterson, Tate Outlon, Tom Hall, Malou Macalinao, Kimberly Fornace, Jennifer Luchavez, Kevin K.A. Tetteh, Gillian Stresman, Alan Kitchen, Peter Chiodini, Fe E. Espino & Chris Drakeley
Stage of publication	**Not yet submitted**

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I performed ELISA experiments with supoort from KP, TO and TH. I also conducted data analyses and wrote the first draft of the manuscript with support from NS and CD.

Student Signature: _____ **Date:** 28/1/2019

Supervisor Signature: _____ **Date:** 25/1/2019

148

Antimalarial antibody detection assays: in search of a standardised tool to confirm the absence of malaria transmission

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Abstract

Background

Antimalarial antibody measurements are unique as they reflect historical and recent exposure to malaria. They may provide additional information in monitoring transmission alongside prevalence of infection in low endemic or pre-elimination settings. Currently, there is no standardised test to detect antimalarial antibodies for epidemiological use. However, standardised commercial enzyme-linked immunosorbent assays (ELISA) are available to screen donor blood products. Here we compare five commercially available ELISA kits (coded A, B, C, D and E) for their relative performance in search of a standardised tool for epidemiological use, with a focus on supporting claims of absence of malaria transmission.

Results

Assay performance was firstly evaluated using serum samples from malaria unexposed individuals as well as *Toxoplasma*-infected individuals. Three out of five kits showed high specificity (98-99%), low cross-reactivity (0-3%) and were considered user-friendly (kit A, B and E). Two kits (A and E), that were still commercially available, were taken forward for epidemiological evaluation. Samples from a low, unstable transmission setting (Praia, Cape Verde; n=1432) and a pre-elimination setting (Bataan, the Philippines; n=2050) were tested. Serological results from kit A overlap with previously described transmission patterns and passively detected case counts in both settings. Results from kit E did not show the expected increase in seroprevalence by age nor an overlap with previously described transmission patterns.

Conclusion

One out of the five commercial ELISA kit was considered applicable for epidemiological use and accurately described transmission patterns in two endemic settings. The use of simple and standardised serological tools to document the absence of malaria transmission can inform control and elimination programs by confirming that regions are free from malaria.

Introduction

A unique property of using antimalarial antibody responses as a measure of transmission is that when combined with age, they reflect a population's exposure history [1–3]. Antibody measures can therefore re-create transmission patterns over time and any fall in, or absence of, antibodies can be interpreted as a decrease in exposure to malaria infections or cessation of transmission. Intuitively, this cumulative metric of exposure to malaria would result in the need for smaller sample sizes to describe transmission at low levels compared to metrics that seek for the proportion of infected individuals in a cross-sectional survey (parasite rate) [4].

Historically, the absence of antibodies in children has been used as proof of cessation of transmission in Greece and Mauritius [5,6]. In these studies, antibody responses to crude parasite extract were determined using an immunofluorescence antibody test (IFAT). More recently, results from Aneityum and Iran suggested absence of transmission by assessing antibody responses to individual recombinant antigens (AMA-1 and MSP-1$_{19}$) [7,8] or schizont extract [7] using an enzyme-linked immunosorbent assay (ELISA). Similar to the historical studies, children showed no antimalarial antibody responses, while some adults did have antimalarial antibodies owing to the persistence of antibodies (and/or memory B cells) once acquired [9–11]. The ELISA platform is considered more objective than IFAT as antibody reactivity is determined by measuring optical density (OD) with a spectrophotometer rather than visual inspection of the strength of fluorescence using a fluorescence microscope [12]. However, at present, there is no standardised ELISA protocol to measure malaria antibodies for epidemiological use: standard operating procedures, positive controls (i.e. hyperimmune sera) and negative controls (i.e. unexposed sera), as well as methods of normalisation vary between research groups and studies, which makes direct comparison of results between countries or populations challenging [4,13].

There are several commercially available ELISA kits, for which production and operating procedures are standardised. These have been used to screen blood donations for evidence of malaria exposure prior to transfusion [14–19]. Some have applied these in an epidemiological context, such as in Ethiopia [20]. To our knowledge, a comparison of the performance of multiple commercially available ELISA kits for epidemiological characterisation of malaria transmission has not been done to date. Therefore, we aimed to compare five commercially available ELISA kits for their applicability and performance in describing malaria transmission. We firstly assessed applicability by comparing assay characteristics such as ease-of-use, specificity, cross-reactivity and the amount of serum needed to test a sample. Secondly, we tested samples from an area of low, unstable malaria transmission (Praia, Cape Verde) and a pre-elimination area (Bataan, the Philippines).

Methods

Study population

Phase I: Assay performance

Assay performance was based on the proportion of samples correctly identified as negative using 223 samples from malaria unexposed UK donors (to assess specificity) as well as 191 samples from *Toxoplasma*-infected, malaria unexposed UK donors (to assess cross-reactivity). Malaria naivety was defined using a questionnaire to exclude malaria risk at the time of donation [14]. *Toxoplasma* was diagnosed with nine commercially available *Toxoplasma* IgG and IgM tests (Supplementary Information I) and was considered positive if it tested positive for any of these tests (J. Newham/A. Kitchen; *unpublished data*). Furthermore, costs, the amount of serum needed to test a sample, and ease-of-use were assessed. For ease-of-us, a composite measure was created based on the number of incubation steps, total incubation time, need for sample preparation and whether reagents were ready-to-use.

Phase II: Epidemiological characterisation

Samples were collected in February 2017 in Bataan, the Philippines (Figure 1a), which saw a steep decline in incidence in the 1990s [21,22] and was declared malaria-free in 2017 (Provincial Epidemiology and Surveillance Unit – Bataan, Malaria Surveillance Report; 2005-2017). Secondly, samples were collected in historical malaria hotspots in Cidade daPraia, Cape Verde (Foton/Tira-Chapéu, Várzea/Taiti and Achada de Santo António; Figure 1b-c) which has seen unstable, low transmission since late 1980s with occasional outbreaks [23–25]. The most recent outbreak started mid-July 2017, with peak cases around the end of August and the end of October [26,27]. The majority of samples in the current study were collected before this outbreak (June-July 2017).

A two-stage cluster randomised sampling design was used with village or a sub-regional administrative unit as primary sampling unit and household as secondary sampling unit. A sample size of 2000 individuals was initially defined for each setting. Under a cross-sectional survey, an entomological inoculation rate of 0.01 and the use of the $MSP1_{19}$ antigen, this sample size was expected to generate a 95% confidence interval for seroprevalence between 4.7% and 6.8% and for the SCR of 0.0029-0.0043 for African settings, and 6.1% and 8.5% and 0.0029-0.0043 for non-African settings [28]. This sample size predicted a power greater than 90% in detecting malaria elimination events occurring at least three years before data collection [29]. For the Cape Verdean setting, staff constraints and study feasibility led to a reduction in the initial sample size to approximately 1,500 individuals. This new

sample size would generate a 95% confidence interval for seroprevalence between 4.5% and 7.0% and for the SCR of 0.0028-0.0044.

All household members over 6 months old who provided consent or assent were included across randomly selected households. A short questionnaire was conducted including demographic information and reported history of malaria. Up to 500µl of whole blood from finger-prick were collected using BD microtainers with EDTA (Becton-Dickinson, Franklin Lakes, New Jersey). Serum was separated at collaborating institutions in-country and were stored at -20°C until shipment on dry ice to the London School of Hygiene and Tropical Medicine. Serum was stored at -20°C until sample processing.

Commercial ELISA kits for antimalarial antibody detection

Five commercial ELISA kits were used according to their instruction manuals (included in **Appendix C**). Kits were coded alphabetically to reduce subjectivity in the interpretation of results (Supplementary Information II). An overview of the standard operating procedure for each of the included kits is shown in Supplementary Table 1. Optical density (OD) measures were read with a spectrophotometer (Dynex® Technologies) at a wavelength of 450nm with a reference filter of 630 nm according to the instruction manuals. OD measures were corrected for blank responses according to kit manuals (hereafter: OD_{corr}).

Statistical analyses

All statistical analyses were performed in RStudio© Version 1.1.456. For Phase I, thresholds for seropositivity were calculated according to instruction manuals. For the epidemiological characterisation in endemic settings (Phase II), only participants with sufficient serum available for all tests (~65 µl) and with age data available were included. Antibody responses from infants under the age of 1 year old were removed due to the possible confounding effects of maternally derived antibodies. In addition to the seropositivity as assigned by the kit instructions, the optimal number of latent serological populations in the antibody data was estimated using mixtures of skewed normal distributions. These distributions include mixtures of normal distributions often used in malaria sero-epidemiological analyses. The optimal number of components was determined by estimating models with one to five components and using Akaike's and Bayesian information criteria for the respective model comparison. If more than one distribution was detected in the data, seropositivity was defined at three standard deviations plus the mean of the distribution with the lowest mean (i.e. the distributions with the lowest mean are assumed seronegatives in the population tested). This analysis was performed using the mixsmsn and sn packages [30,31]. A simple reversible catalytic model was

fitted to the seroprevalence and age data for each endemic setting using maximum likelihood methods [2,3]. The model generates a seroconversion rate (SCR) which reflects the rate at which a population becomes seropositive and is an indication of the force of infection, and seroreversion rate (SRR) which reflects the rate at which the population reverts to seronegative. This model was used to generate seroconversion curves (i.e. age seroprevalence curves). If visual examination of the seroconversion curve suggested that a change in transmission had occurred, a model with two SCRs was fitted to determine when the change most likely had occurred as described previously [1,7]. A likelihood ratio test was used to determine if the model allowing for two SCRs fitted the data better than the model allowing for one SCR ($p < 0.05$). Furthermore, age-antibody curves using continuous antibody response data were assessed using the *tmleAb* R package described by Arnold et al. [32]. In short, mean antibody responses (OD_{corr}) were modelled as a function of age using a super learner algorithm in which an ensemble of models and algorithms are used. The ensemble included: the simple mean, generalized linear models, generalized additive models with natural splines, locally weighted regression (lowess) and the recently described antibody acquisition model with constant rates [33]. Coefficients representing the weights for each algorithm were recorded. Logistic regression analysis was done to identify demographic and household factors associated with seropositivity in each setting. An adjusted model was created with factors testing statistically significant in univariate analysis (p-value < 0.05).

Ethical approval

Ethical approval to test anonymised UK donor samples collected by Public Health England/NHS Blood and Transplant was obtained through the LSHTM Research Ethics Committee (11684). For the surveys in Cape Verde and the Philippines, ethical approval was obtained through the LSHTM Research Ethics Committee (11684), the Comissão de ética nacional em Pesquisa da Saúde in Cape Verde (65/2016) and the Research Institute for Tropical Medicine in the Philippines (2016-26). All survey participants provided written informed consent or assent.

Results

Phase I: Assay performance

Manuals from commercial kits report sensitivities ≥93% and specificities ≥96% (Table 1). The proportion of samples correctly identified as negative either for malaria unexposed individuals or those with other infections (*Toxoplasma*-positive) was high across all kits (>96%) except for kit D (81% and 84%, respectively). Costs per sample were highest for kit C, D and E (≥£1.71 compared to ≤£1.32 for kit A and B), while kit C needed the highest volume of serum (150 µl compared to ≤50 µl for the

other kits) and was considered least user-friendly (Table 2 and Supplementary Table 1). Based on these results, three commercial kits were taken forward to compare their use for epidemiological characterisation (kit C and D were dropped). However, commercial production of kit B was discontinued after finalisation of Phase I and it was therefore unavailable; thus, only kit A and E were taken forward.

Phase II: Epidemiological characterisation

After aliquoting serum from Eppendorf tubes into deep well plates, samples were processed over two days for Cape Verde (sixteen 96-well plates) and 3 days for the Philippines (twenty-two 96-well plates). In Cape Verde, 1396 out of 1432 samples collected were available for analyses (no age data available or younger than 1 year old, n=33; not enough serum available for all tests, n=11). In the Philippines, 1824 out of 2050 collected were available (no age data available or younger than 1 year old, n=11; not enough serum available for all tests, n=179; participant lived outside main study area, n=36).

Antibody metrics for transmission intensity

Kit E did not show the expected increase in age-specific seroprevalence and seroconversion curves did not reflect known (historical) transmission patterns (Supplementary Information III). For kit A, depending on the threshold used for seropositivity (detection of latent distributions within the antibody data recorded or according to kit manual; see Supplementary Figure 1), seroprevalence ranged from 15.4 (95% CI: 13.7% – 17.1%) to 18.6% (16.9% – 20.5%) in Bataan, the Philippines and 4.8% (3.7% – 6.1%) to 6.5% (5.3% – 7.9%) in Praia, Cape Verde. Hereafter, only results using seropositivity according to the distribution approach will be presented in text but results for both are presented in figure legends.

In Bataan, the Philippines, change points in the seroconversion curves were estimated at 21 (21 – 21; Figure 2c), which coincides with a sudden drop in reported cases at local health facilities (Figure 2a). The estimate of the recent SCR was 0.0002 (0.0001 – 0.0016) and an overall SRR of 0.0113 (0.0005 – 0.0188) year^{-1}. Age-antibody curves using continuous antibody response data (i.e. OD$_{corr}$), showed a similar pattern to the seroconversion curve (Figure 2c). Seroprevalence in those born after this decrease in transmission was minimal, 0.6% (5/878), they did not report history of malaria and the median age was 15 (9 – 20). In individuals aged 37 years and older (n=534), seroprevalence was higher in men (48.4% compared to 31.9% in women, Chi squared test p<0.001; Figure 2d).

In Cape Verde, both historical and recent transmission were low as represented by the seroconversion curve (Figure 3b) which is reflected in the low case counts since the late 1980s (Figure 3a). The SCR was estimated at 0.0018 (0.0013 – 0.0026) and SRR at 0.0000 (0.000 – 0.0156) year^{-1}, while no

statistically significant change point was observed. However, age-antibody curves showed a peak in antibody responses around 30-year olds as well as 45 to 50-year olds (Figure 3c). Seroprevalence by gender reveals that seropositivity is higher in men aged 30 to 46 (10.1%, 8/79) compared to women (0.5%, 8/176) although this difference was not statistically significant (Fisher's Exact test p=0.100).

Associations with seropositivity

In the Philippines, adjusted logistic regression analysis of factors associated with seropositivity for kit A identified: adults, males, bed net use and self-reported history of malaria (Table 3). In Cape Verde, factors associated with seropositivity were younger children (1 to 5 year olds), no use of preventions for bites, bed net use and self-reported history of malaria.

Discussion

There is historical evidence that antibody detection may help in certifying areas malaria-free [5,6], but there is no standardised approach available. The application of a standardised assay to detect antimalarial antibodies is an essential step in the evaluation of the use of serological data to support claims of absence of transmission. Commercially available ELISA kits undergo rigid standardisation processes and have been applied to screen blood products prior to donation to minimise risks of transfusion-transmitted malaria [14–19]. Here, we compared five commercial ELISA kits for their applicability (Phase I) and performance (Phase II) in epidemiological characterisation of malaria transmission at low endemicity and pre-elimination.

Kit C and D were not considered applicable for use in epidemiological surveys owing to relatively high costs per sample (kit C, D and E ≥£1.71 compared to ≤£1.32 for kit A and B) and low ease-of-use due to longer incubation times (150 min and 135 min; compared to 105 min for kit E and 90 min for kit A and B), the need for sample preparation, the need for reagent preparation and an additional incubation step for kit C. Although kit E also required samples to be prepared, all reagents were ready-to-use; and while kit A needed one reagent to be prepared, samples did not need preparation, and incubation steps and time were least compared to other kits. In addition, kit C showed relatively low specificity (82% compared to ≥98% for the other kits) and high cross-reactivity (16% compared to ≤2% for the other kits), while it required the most serum to run a sample (150 µl compared to ≤50 µl for the other kits). High specificity and low cross-reactivity are essential in the use of any assay, especially in the context of determining the absence of transmission (i.e. minimizing the risk of false-positives) [34]. Likewise, sensitivity is an important factor, which we did not test for in this study. However, instruction manuals report sensitivities ≥93%, while manuals for kit A and B also included the number

of samples tested for their assessment of sensitivity (13,608 and 450 patient samples). Sensitivity as assessed in other research studies is discussed below. Finally, the amount of serum needed per sample is an important factor as most malaria transmission surveys use finger-prick blood [35] and ideally, sufficient sample would be collected from each participant to be able to repeat the assay if needed. Therefore, 150 µl of serum was not considered feasible (kit C). Based on these findings, kit A and B were considered most appropriate for use in large-scale epidemiological surveys, followed by kit E (owing to higher costs per sample and relatively long incubation times compared to kit A and B but an otherwise similar performance). Unfortunately, commercial production of kit B was discontinued after finalisation of Phase I.

To assess the performance of kit A and E in epidemiological characterisation, samples from Bataan, the Philippines (declared malaria-free in 2017) and Praia, Cape Verde (unstable, low transmission) were tested. Results from kit A described historical and recent malaria transmission in Bataan accurately, both for metrics using seropositivity and for antibody density over age. Seroconversion curves showed a change in the force of infection which coincides with a sudden decrease in passively detected malaria cases in 1995 [21] following the roll-out of community-based volunteers to improve access to malaria diagnosis in 1994 [22]. Seroprevalence in those born since this sudden decrease in transmission was low (i.e. 0.6%). Whether these represent false-positive results or true responses following asymptomatic, low-density infections or infections acquired outside the study area, is unknown. The recent SCR approximated zero with ~0.02% of the population seroconverting, which is lower than those recorded in Sri Lanka during pre-elimination using a research-based ELISA protocol [36]. For Cape Verde, the low levels of transmission over the past decades were correctly identified as shown by the low, constant SCR recorded by kit A (i.e. ~0.18% of the population seroconverts per year). The age-antibody curve revealed a peak in antibody titre around 30- and 45-50-year olds and increased seroprevalence was seen in men, possibly due to work-related risk of malaria infection (i.e. work involving night-shifts) or travel-related risk (though this was not statistically significant). This pattern has previously been described for passively collected malaria case counts recorded between 2007-2009, as two thirds of the reported cases were in adult men [23]; and for those recorded between 2010-2016 as 77% of the cases were in males and 82% of the cases in individuals older than 20 years of age [30]. Importantly, an outbreak of malaria occurred in Cape Verde in the final weeks of sample collection for the current study [26]. Both kits (A and E) detect IgM as well as IgG, which is an added advantage over most research-based protocols. As IgM is an early responder following malaria re-infection [37,38], separately assessing IgG and IgM may help in distinguishing recent compared to historical transmission more accurately. Results recorded by kit E did not show the expected increase in age-specific seroprevalence and seroconversion curves did not reflect malaria transmission patterns

based on passively collected malaria case counts. It was therefore not considered appropriate for epidemiological characterisation of transmission.

Many studies have described the overlap in serological results with other malaria metrics using a research-based ELISA, detecting antibodies to asexual parasite antigens in endemic settings [1–4,39]. To our knowledge, only two other studies have used a commercially available ELISA kit for epidemiological characterisation of malaria in an endemic settings [20,40]. Birhanu et al. assessed the parasite rate by microscopy and RDT alongside antimalarial antibody detection by kit A in Ethiopian children aged 2-9 years old. They showed a parasite rate of zero percent in the majority of study sites while seroprevalence differed considerably (1.6 – 55.7%) [20]. In Iran, Kit D was used to test samples from a hypoendemic region and it recorded seroprevalence at <1% in those aged 20 years or younger. All participants were microscopy negative and had no circulating HRP2 antigen [40]. In contrast, many have assessed the test performance (i.e. sensitivity/specificity) of commercial ELISA kits using samples from malaria patients or those without malaria risk (i.e. no travel to endemic settings). Alongside kits not assessed in this study, kit D has shown sensitivities ranging from 71% using IFAT as the gold standard [41] and 83% [42] to 91% [15] using microscopy as the gold standard. Specificities ranged from 85% using microscopy as the gold standard [42] and 81% as reported by us to 92% [15] if samples from individuals who had not travelled to malaria endemic regions were tested. Overall sensitivities and specificities were lower than those reported by the instruction manual for kit D (94% and 100%, respectively). However, the number of samples was limited in some of these studies (n=11 for sensitivity [15]) and sampling approaches varied (i.e. testing suspected malaria patients [42] compared to healthy subjects [15] and confirmed malaria patients [15,41]). Thongdee et al. assessed the sensitivity and specificity of kit E by testing samples from patients with malaria, patients with fever related to other infections and healthy subjects in Thailand [43]. Using malaria diagnosis by microscopy as the reference, the sensitivity of kit E was 89% (81 – 95%) and the specificity was 92% (86 – 95%) – lower than those reported in the instruction manual (96% and 98%, respectively) as well as the specificity reported in this study (98%).

Although the current study did not test all currently available commercial ELISA kits for antimalarial antibody detection, it is promising that one of the five kits accurately described transmission patterns in two endemic settings. As costs are an important factor for wide-scale use, it would be advantageous if these could be brought down for kit A (currently £1.30/sample excluding laboratory capital expenses and technician time). There are some technical advantages of kit A over research-based antibody detection protocols. For example, blocking of non-specific binding to malaria antigens in kit A seems extremely efficient, demonstrated by the fact that although neat serum is tested, little to no background responses are seen (i.e. seroprevalence in those born since the absence of passively

detected locally acquired cases in Bataan was 0.6%). Although research-based ELISA protocols dilute serum concentrations to 1 in 100s [36] or 1000 [1,3,7,35], which enables use of dried blood spots, recent SCRs were higher in Sri Lanka during pre-elimination than those recorded in Bataan in the current study [36]. Nevertheless, collecting dried blood spots compared to (neat) serum samples has both practical and logistical advantages in field surveys [35]. Ideally, commercial ELISA kits would be optimised for use of elution of dried blood spots, or research-based protocols would increase the serum concentration while aiming to maintain or reduce non-specific binding.

Conclusion

One commercial ELISA kit was considered applicable for large-scale use in epidemiological surveys and accurately described malaria transmission in a low transmission and pre-elimination setting. Commercial ELISA kits detecting antimalarial antibodies may be of use to certify areas malaria-free in combination with other metrics such as (passively detected) case counts.

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Table 1: Assay characteristics for five commercial enzyme-linked immunosorbent assays for antimalarial antibody detection (A-E) according to instruction manuals.

	A	B	C	D	E
Antigenic targets	Four recombinant antigens for four species	Recombinant antigens for four species	Recombinant antigens for all *P.* species	Recombinant antigens for four species	CSP and MSP1 proteins from *Pf* and *Pv*
Subclasses	IgG, IgM, IgA	Not reported	IgG, IgM, IgA	Not reported	IgG, IgM
Samples/plate*	91	91	89	92	91
Duration, total incubation time in min	90 min	90 min	150 min	135 min	105 min
Specificity - all species (samples tested)	96% (n=13608)	100% (n=450)	>98% (Not reported)	100% (Not reported)	98% (Not reported)
Sensitivity - all species (samples tested)	Not reported	98% (n=528)	>95% (Not reported)	94% (Not reported)	96% (Not reported)
Sensitivity - *Pf* only (samples tested)	93% (n=76)	98% (n=410)	Not reported	Not reported	Not reported

*I.e. all kits use 96-well plates and require a certain number of controls to be run alongside samples.

Table 2: Costs per sample, amount of serum needed to run a sample, ease-of-use, specificity and cross-reactivity for five commercial enzyme-linked immunosorbent assays for antimalarial antibody detection (A-E).

	A	B	C	D	E
Costs/sample[a]	£1.30	£1.32	£1.84	£2.09	£1.71
Amount of sample	50 µL	50 µL	150 µL	2 µL	10 µL
Ease-of-use[b]	High	High	Low	Medium	High
Proportion negative if:					
Malaria unexposed (n=223)	99% (97-100%)	99% (97-100%)	98% (95-99%)	81% (75-86%)	98% (95-100%)
Toxoplasma-infected (n=191)	100% (98-100%)	99% (96-100%)	100% (98-100%)	84% (78-89%)	98% (95-99%)

[a] Costs per sample are based on running a full 96-well plate of samples after adding necessary controls according to kit instruction manuals (see Table 1). Commercial assays were bought in bulk (i.e. 25 plates per brand) in January 2016 for Phase I and March 2017 for Phase II. Costs shown here are based on the most recent prices from 2017 for assay A, B and E. [b] Ease-of-use was assessed based on the number of incubation steps, incubation time, need for sample preparation and whether reagents are ready-to-use (summarised in Supplementary Table 1).
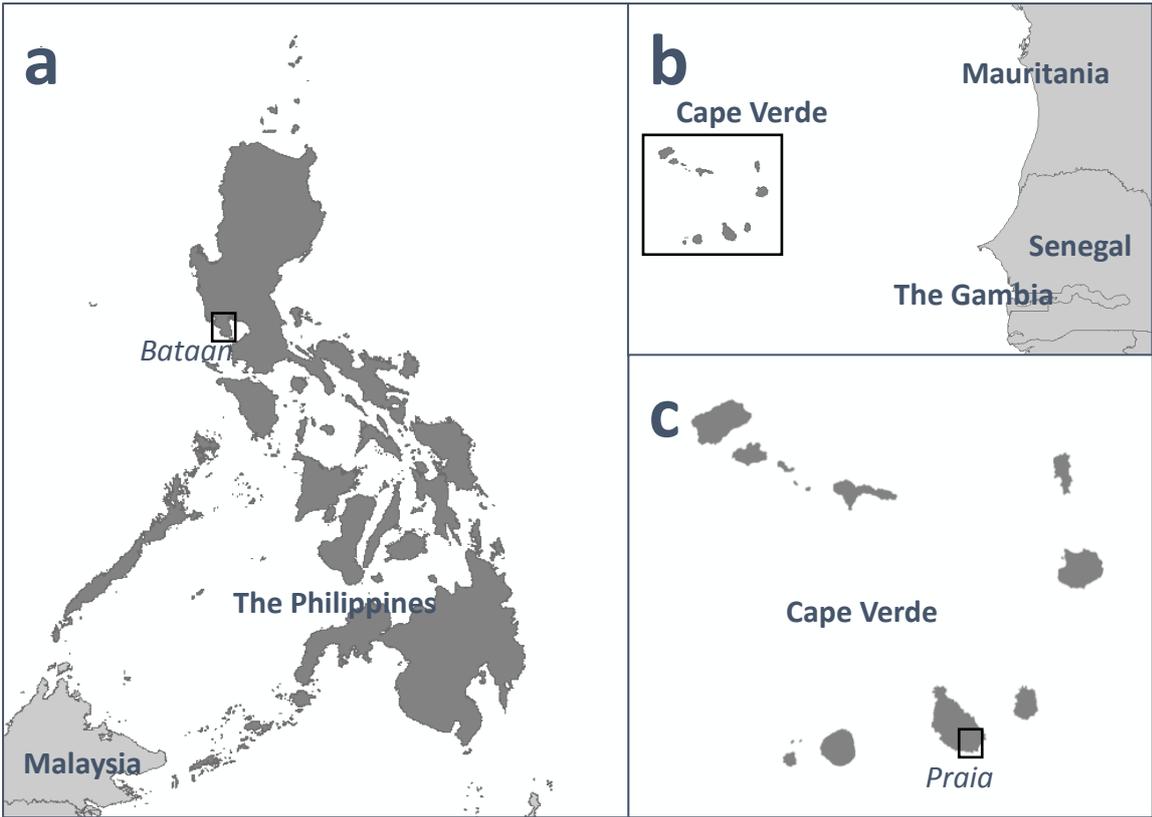
Figure 1: Map of survey locations in Bataan, the Philippines (a) and Praia, Cape Verde (b-c). The square highlighting Cape Verde in (b) is enlarged in (c).
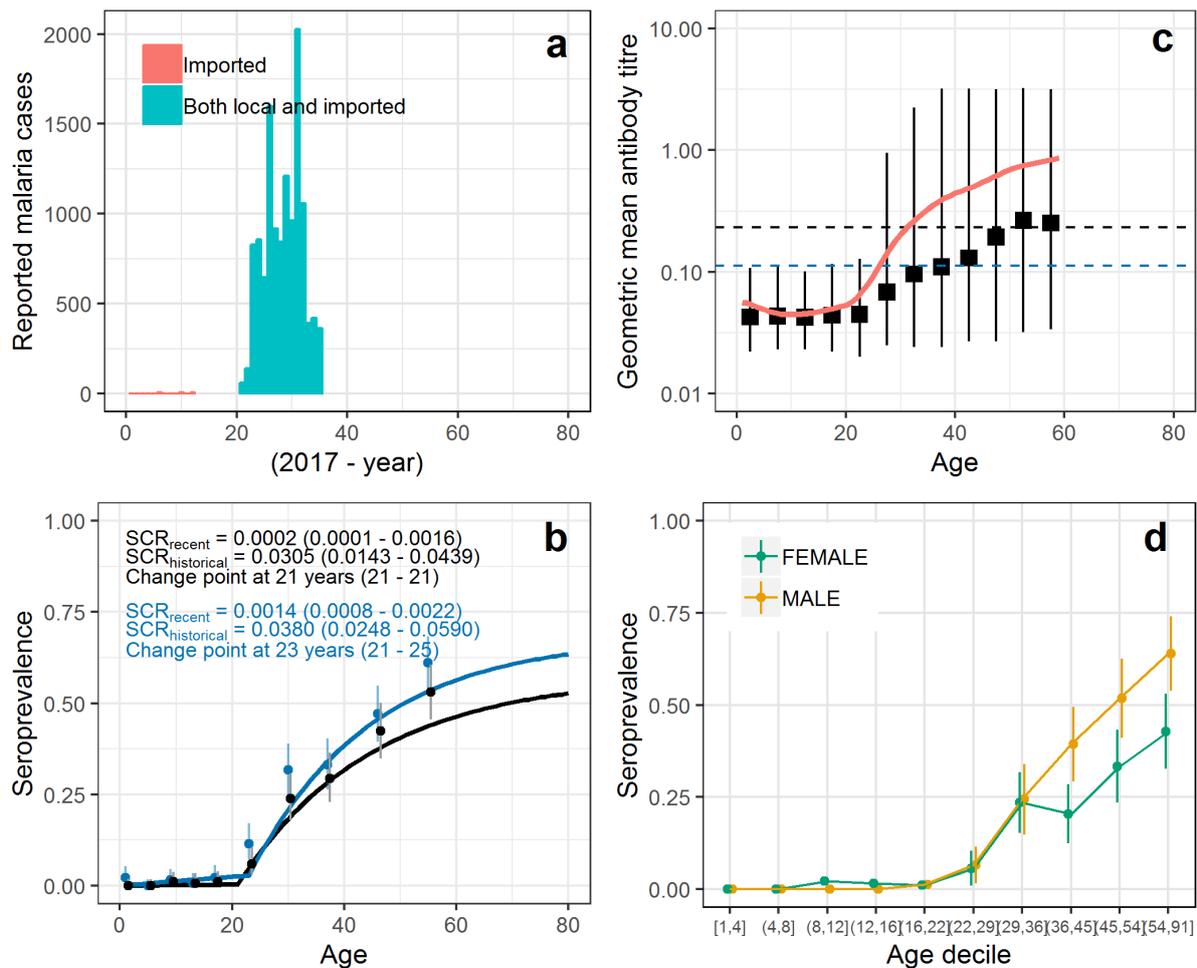
Figure 2: Reported malaria cases (a) and antibody metrics using antibody responses recorded by enzyme-linked immunosorbent assay A (b-d) in Bataan, the Philippines. In (**a**) counts of reported malaria cases at local health facilities in Bataan according to the source (local or imported) are shown over time; data was available for 0 to 12 years prior to data collection (i.e. 2017 − 2005; Provincial Epidemiology and Surveillance Unit - Bataan, Malaria Surveillance Report) and 21 to 35 years prior to data collection (i.e. 1996 − 1982 [21]). In (**b**) seroconversion curves of seroprevalence by age are shown; solid lines represent the fit of the reversible catalytic model [2], while dots represent observed seroprevalence estimates with 95% confidence intervals in vertical segments. Thresholds for seropositivity were calculated from the mean of the lower distribution of antibody responses plus 3 standard deviations using mixtures of skewed normal distributions (black) or according to the kit instruction manual (blue). In (**c**) boxplots of geometric mean antibody titre (i.e. optical density corrected for blank responses) are shown over bins of 5 years of age for those aged 60 years or younger (range of bin size 63 − 268). The red line represents the age-antibody fit using a super learner algorithm as previously described by Arnold et al. [32]: coefficients representing the weight of each contributing algorithm were 0.51 for generalized additive model, 0.43 for locally weighted regression and 0.06 for the generalized linear model. Horizontal dashed lines represent thresholds for

seropositivity according to the mean of the lower distribution of antibody responses plus 3 standard deviations using mixtures of skewed normal distributions (black) or according to the instruction manual (blue). In (**d**) seroprevalence is shown by age deciles and gender; dots represent observed seroprevalence estimates with 95% confidence intervals in vertical segments. SCR: seroconversion rate.
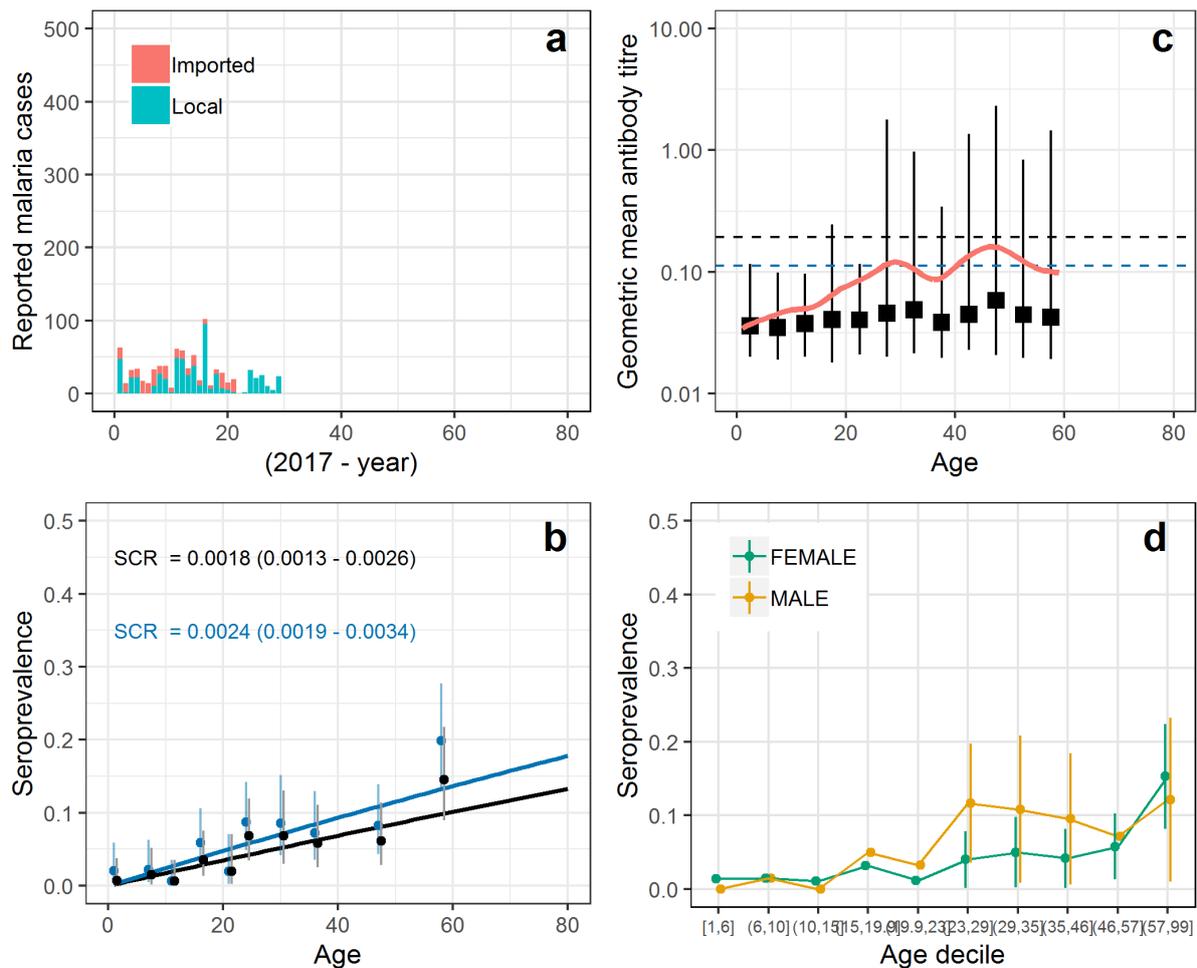
Figure 3: Reported malaria cases (a) and antibody metrics using antibody responses recorded by enzyme-linked immunosorbent assay A (b-d) in Praia, Cape Verde. In (**a**) counts of reported malaria cases in Praia according to the source (local or imported) are shown over time, data was available from 1 to 21 years prior to data collection (i.e. 2016 – 1996) and 22 to 31 years prior to data collection (i.e. 1995 – 1986; counts from 1986 – 1987 are for Santiago island [23]). In (**b**) seroconversion curves of seroprevalence by age are shown; solid lines represent the fit of the reversible catalytic model [2], while dots represent observed seroprevalence estimates. Thresholds for seropositivity were calculated from the mean of the lower distribution of antibody responses plus 3 standard deviations using mixtures of skewed normal distributions (black) or according to the kit instruction manual (blue). In (**c**) boxplots of geometric mean antibody titre (i.e. optical density corrected for blank responses) is shown over bins of 5 years of age for those aged 60 years or younger (range of bin size 51 – 169). The red line represents the age-antibody fit using a super learner algorithm as previously described by Arnold et al. [32]: coefficients representing the weight of each contributing algorithm were 0.67 for the generalized additive model, 0.31 for the generalized linear model and 0.02 for the antibody acquisition model with constant rates. Horizontal dashed lines represent thresholds for seropositivity according to the mean of the lower distribution of antibody responses plus 3 standard deviations using

mixtures of skewed normal distributions (black) or according to the instruction manual (blue). In (**d**) seroprevalence is shown by age deciles and gender; dots represent observed seroprevalence estimates with 95% confidence intervals in vertical segments. SCR: seroconversion rate.

Table 3: Logistic regression analysis of explanatory factors for seropositivity as recorded by enzyme-linked immunosorbent assay A in Praia, Cape Verde and Bataan, the Philippines.

	Cape Verde					Philippines**				
Distribution pos	n/N	Unadjusted	p	Adjusted	p	N	Unadjusted	p	Adjusted	p
Area										
1	26/492	1				35/241	1			
2	27/550	0.93 (0.53 - 1.62)	0.783			101/465	1.20 (0.80 - 1.84)	0.395		
3	14/354	0.74 (0.37 – 1.41)	0.370			82/563	1.00 (0.66 - 1.56)	0.988		
4						62/423	1.01 (0.65 - 1.60)	0.962		
Age group										
1-5	1/116	0.12 (0.19 - 0.96)	0.039	9.14 (2.82 – 56.09)	0.002	0/236	NA	0.967	NA	0.967
6-15	3/319	0.14 (0.16 - 0.54)	0.001	1.33 (0.06 – 14.15)	0.815	3/475	0.02 (0.00 – 0.05)	<0.001	0.02 (0.01 – 0.06)	<0.001
>15	63/961	1		1		277/1113	1		1	
Gender										
Female	39/860	1				121/929	1		1	
Male	28/536	1.16 (0.70 - 1.90)	0.558			159/895	1.44 (1.12 – 1.87)	0.005	1.56 (1.17 – 2.07)	0.003
Travel Yes	9/77	1					NA			
No	58/1250	0.40 (0.20 – 0.89)	0.014							
IRS										
No	46/936	1				234/1551	1			
Yes	20/414	0.98 (0.56 - 1.66)	0.948			46/273	1.14 (0.80 – 1.60)	0.456		
Prevention bites										
Repellents	20/311	1.10 (0.59 – 2.02)	0.768	0.99 (0.51 – 1.89)	0.979	126/898	1.23 (0.95 – 1.59)	0.117		
Other	20/620	0.53 (0.29 – 0.98)	0.042	0.54 (0.29 – 1.00)	0.050	149/892	1.06 (0.35 – 2.56)	0.912		
None	24/407	1		1		5/29	1			
Sleep under bed net last night No	62/1360	1		1		130/979	1		1	
Yes	4/34	2.79 (0.81 – 7.35)	0.119	3.60 (0.95 – 10.74)	0.034	150/845	1.41 (1.09 – 1.82)	0.008	1.84 (1.38 – 2.46)	<0.001
Malaria history*										
No	59/1369	1		1		222/1725	1		1	
Yes	7/25	8.63 (3.25 – 20.66)	<0.001	6.60 (2.42 – 16.36)	<0.001	56/87	12.23 (7.77 – 19.59)	<0.001	7.41 (4.61 – 12.14)	<0.001
Antimalarial in last 2 weeks No	62/1370	1					N/A			
Yes	3/21	3.52 (0.81 – 10.74)	0.048							

CV: 1: Varzea; 2: Tira Chapea/Fonton, 3: ASA. Philippines: 1: Laplap; 2: Minanga; 3: Proper; 4: Samuyao. *Youngest reported in Cape Verde 16 years old (range 16-66) and in the Philippines 10 years old (10-87). **Removed 36 samples in other areas than main study area.

Supplementary Tables

Supplementary Table 1: Standard operating procedures for five commercial enzyme-linked immunosorbent assays for antimalarial antibody detection (A-E) and composite measure of ease-of-use. RT: room temperature; min: minutes.

	A	B	C	D	E
Sample volume (Dilution)	50 µl (neat)	50 µl (neat)	150 µl (3:4)	2 µl (1:100)	10 µl (1:101)
Incubation, min (Temperature)	30 (37°C)	30 (37°C)	60 (37°C)	60 (RT)	60 (37°C)
Wash 1, n	5	5	4-5	4	3
Add conjugate	50 µL (prepare)	50 µL	#1: 150 µL (prepare) #2: 100 µL	100 µL (prepare)	100 µL
Incubation, min (Temperature)	30 (37°C)	30 (37°C)	#1: 30 #2: 30 (37°C)	60 (RT)	30 (RT)
Wash 2, n	5	5	4-5	4	3
Add substrate	50 µL	50 µL	200 µL	100 µL (prepare)	100 µL
Incubation (Temperature)	30 (RT)	30 (RT)	30 (RT)	15 (RT)	15 (RT)
Add stop	50 µL	50 µL	100 µL	50 µL	100 µL
Read plate	450nm (reference 630nm)	450nm (reference 630nm)	450nm (reference 630nm)	450nm (blank on air)	450nm (reference 630nm)
Ease-of-use	High	High	Low	Medium	High
- Sample preparation	No	No	Yes	Yes	Yes
- Incubation steps	3	3	4	3	3
- Incubation time, min	90	90	150	135	105
- Ready-to-use reagents (n/N)	2/3	3/3	3/4	1/3	3/3

Supplementary Figures
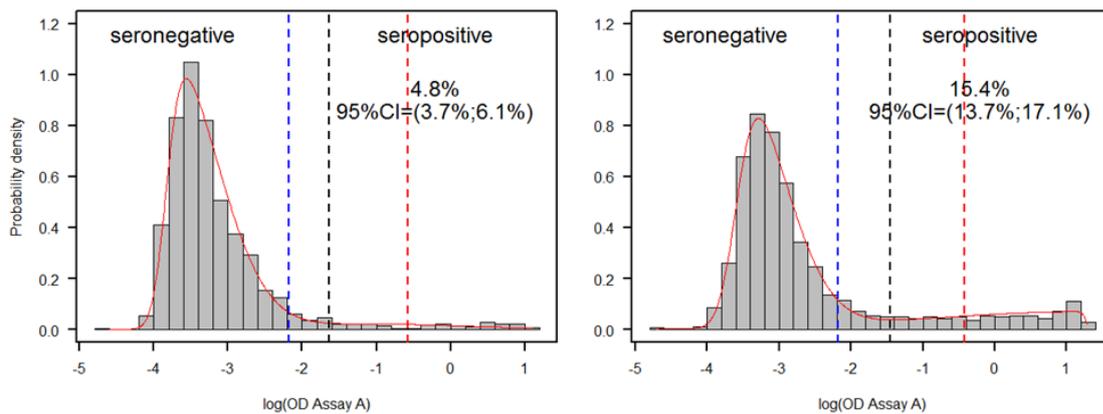
Supplementary Figure 1: Histogram of antibody responses in Praia, Cape Verde (left) and Bataan, the Philippines (right) as recorded by enzyme-linked immunosorbent assay A as well as thresholds for seropositivity according to different methods. The optimal number of latent serological populations in the antibody data was estimated using mixtures of skewed normal distributions. For both settings, the optimal number of components was two using Akaike's and Bayesian information criteria. Solid red lines represent fitted density distributions. Dashed vertical lines represent thresholds for seropositivity according to the instruction manual (blue), or the mean of the lower distribution plus three (black) or five (red) standard deviations (SD). Seroprevalence estimates with 95% confidence intervals (CI) using the three SD approach are shown on plots.

Supplementary Information I: *Toxoplasma* diagnosis

Toxoplasma was diagnosed with nine commercially available *Toxoplasma* IgG and IgM tests. All 191 serum samples were tested on:

- Microgen Mercia Toxoplasma IgG
- Microgen Mercia Toxoplasma IgM
- Biorad Platelia Toxoplasma IgG
- Biorad Platelia Toxoplasma IgM
- Access Toxoplasma IgG
- Access Toxoplasma IgM
- Mast Diagnostics Mastafluor Toxoplasma IgG
- Mast Diagnostics Mastafluor Toxoplasma IgM
- Euroimmune Toxoplasma IgG ELISA
- Euroimmune Toxoplasma IgG IFT
- Euroimmune Toxoplasma IgM ELISA
- Euroimmune Toxoplasma IgM IFT
- Biokit Toxocell Latex
- Abbott Architect Toxoplasma IgG
- Abbott Architect Toxoplasma IgG Avidity
- Abbott Architect Toxoplasma IgM
- Diasorin Liason Toxoplasma IgG II
- Biomerieux Vidas Toxoplasma IgG II
- Biomerieux Vidas Toxoplasma IgG Avidity
- Biomerieux Vidas Toxoplasma IgM
- Biomerieux Vidas Toxoplasma Competition

A sample was considered positive if it tested positive for any of these tests. A subset of samples was confirmed by the Sabin Feldman dye test and the IgM immunosorbent agglutination assay at the Swansea Toxoplasma Reference Laboratory.

J. Newham/A. Kitchen; *unpublished data*

Supplementary Information II: Included commercial ELISA kits (A-E)

Five commercially available ELISA kits for antimalarial antibody detection were evaluated in this study (Table SII.1). Instruction manuals of these kits are included in Appendix C.

Table 1: ID (A-E) and brand names for five commercial enzyme-linked immunosorbent assays for antimalarial antibody detection.

Kit	Name
A	Trinity Biotech
B	NewBio
C	DiaPro
D	Cellabs
E	NovaTec / NovaLisa

Supplementary Information III: Epidemiological characterisation using kit E

Data analyses were performed in STATA version 15. Antibody levels recorded by kit E were similar across age groups and thus did not show the expected increase over age in both Praia, Cape Verde and Bataan, the Philippines (Figure SIII.1). This was reflected in seroprevalence as recorded responses all existed around the threshold for positivity. This resulted in high seroprevalence in Cape Verde for all ages and for children in the Philippines (Table SIII.1 and Figure SIII.2), while transmission patterns as recorded by locally reported malaria cases suggest low transmission in Cape Verde (Figure 3a) as well as an absence of transmission in recent years in the Philippines (Figure 2a).

Table 1: Seroprevalence as recorded by enzyme-linked immunosorbent assay E in Praia, Cape Verde and Bataan, the Philippines (overall and by age category). Seropositivity was determined according to the kit instruction manual.

	Philippines	Cape Verde
Overall	11%	51%
Age category, years:		
1/5	4% (10/235)	30% (35/116)
6/15	10% (47/479)	48% (153/319)
>15	14% (154/1132)	54% (524/961)
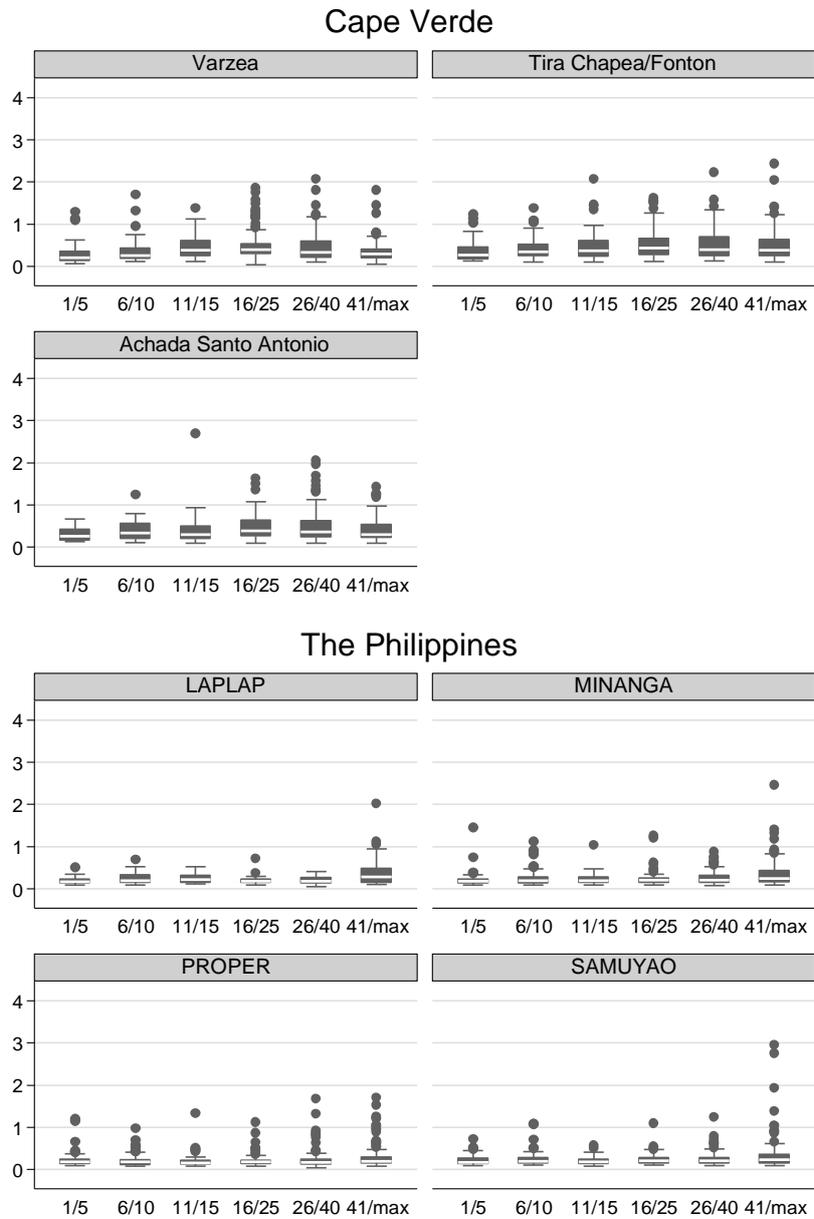
Figure 1: Boxplots of antibody levels recorded by enzyme-linked immunosorbent assay E over age categories and sub-regions in Praia, Cape Verde and Bataan, the Philippines. OD: Optical Density corrected for blank responses.
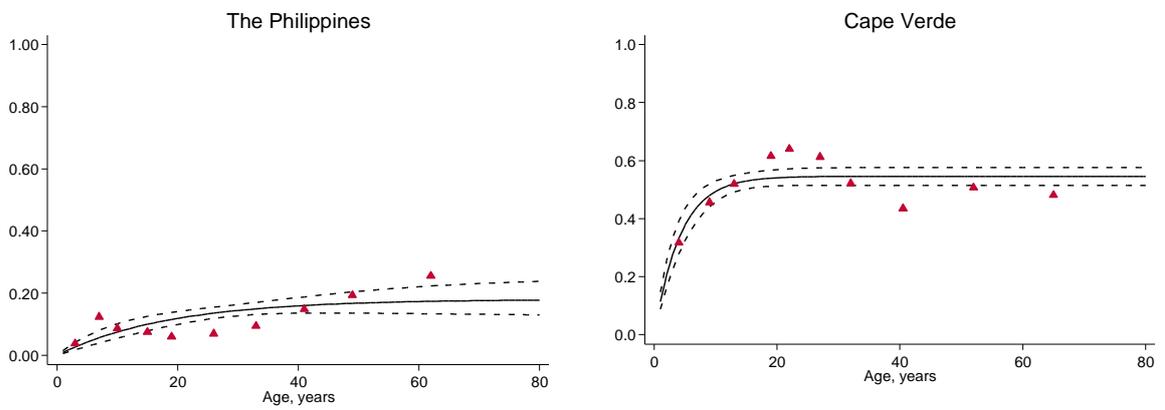
Figure 2: Seroconversion curves using seropositivity as recorded by enzyme-linked immunosorbent assay E in Praia, Cape Verde and Bataan, the Philippines. Seropositivity was determined according to the kit instruction manual.

Additional File: Application and performance of a research-based ELISA protocol in which antigens were pooled in epidemiological characterising of malaria transmission patterns at low transmission and pre-elimination

In **Chapter 6** results for the epidemiological application of five commercially available ELISAs are discussed (i.e. ease-of-use, specificity, cross-reactivity, the volume of serum needed to run an experiment and costs). In addition, their performance in characterising transmission in a low transmission (Praia, Cape Verde) and pre-elimination (Bataan, the Philippines) setting was shown. In this addition, these results are presented for a research-based ELISA protocol in which antigens were pooled.

Methods

Samples from malaria unexposed individuals as well as *Toxoplasma*-infected individuals were tested. Furthermore, samples from a low, unstable transmission setting (Praia, Cape Verde; n=1432) and a pre-elimination setting (Bataan, the Philippines; n=2050) were tested. For details on study populations and sample collection see **Chapter 6**.

A previously described research-based ELISA protocol [1] was performed with the following modifications (SOP included in **Appendix D**). To optimally capture exposure, a pool of five *P. falciparum* antigens was used: AMA-1, MSP-1$_{19}$, MSP-2 Dd2, MSP-2 CH150/9 and GLURP-R2. To increase throughput and reduce costs, samples were run in single. A positive control standard curve using a Tanzanian hyperimmune sera pool was run in duplicate on every plate starting at 1:10 with a 5-fold dilution over six steps and samples were tested at a final concentration of 1:1000. Optical density (OD) measures were read with a spectrophotometer (Dyson Technologies) at a wavelength of 450nm. OD measures were corrected for blank responses (hereafter: OD$_{corr}$). As standard curves of hyperimmune sera did not saturate at the bottom asymptote, these could not be used to adjust OD$_{corr}$ measures for plate-to-plate variation. Seropositivity for samples from endemic settings was identified as described in **Chapter 6** for commercial ELISA kit results (i.e. the *sn* package in R).

Results

The time to run the assay was considerably longer for the research-based ELISA compared to commercially available ELISAs (Table 1). Costs and the amount of serum needed to run an experiment were minimal for the research-based ELISA (Table 2). Seroprevalence results for malaria unexposed (i.e. representing specificity) and *Toxoplasma*-infected (i.e. representing cross-reactivity) individuals

are not shown as thresholds for seropositivity could not be defined. Thresholds for seropositivity in the research-based ELISA are usually based on finite mixture models for endemic populations whereby two distribution are identified, and the lower distribution is assumed to be formed by seronegatives. The mean of the lower distribution plus three standard deviations is then used as the threshold for seropositivity. As the malaria unexposed individuals and *Toxoplasma*-infected individuals should not show antimalarial antibodies, only one distribution could be identified. Another approach is to use the mean plus three standard deviations of antibody responses from an unexposed population. As the malaria unexposed individuals and *Toxoplasma*-infected individuals are such populations, by default the ~2.5% outliers on the right-hand side of the normal distribution would therefore be identified as seropositive.

Scatter plots of OD_{corr} measurements recorded by the research-based ELISA and the commercial ELISA kit A show minimal correlation between the two assays (Figure 1). In Cape Verde and the Philippines two mixtures were estimated, however both distributions were assumed be seronegative in Cape Verde (i.e. mean OD_{corr} values for these distributions were 0.04 and 0.14) and the Philippines (0.105 and 0.293), Figure 2. Therefore, seroprevalence and seroconversion curves could not be estimated.

Conclusion

The research-based assay was not optimised sufficiently as it did not identify negative and positive populations accurately (Figure 1 and 2). This may be due to insufficient optimisation of antigen and serum concentrations as well as insufficient blocking efficiency for non-specific binding on the research-based ELISA. Therefore, differentiation of individuals exposed and unexposed to malaria was not possible in both Praia, Cape Verde and Bataan, the Philippines. As shown in **Chapter 6**, commercial ELISA kit A was able to identify a seropositive population in both settings and serological results overlapped with transmission patterns assessed through passively detected case counts. However, ELISA kit A is relatively expensive compared to the research-based ELISA (Table 2) and the antigens tested for are unknown. Although the overall duration of the research-based ELISA protocol is ~48 hours, this includes 32 hours incubation and 40 plates can be run per experiment thus technician time per plate is similar to commercial ELISAs. Therefore, if the research-based ELISA could be improved, the low costs and ability to adjust antigen pools for specific settings and/or use-case scenarios would be advantageous compared to commercial ELISAs.

References

1. Corran PH, Cook J, Lynch C, Leendertse H, Manjurao A, Griffin J et al. Dried blood spots as a source of anti-malarial antibodies for epidemiological studies. Malaria J. 2008;7:195.

Table 1: Assay characteristics for five commercial (A-E) and the research-based enzyme-linked immunosorbent assays for antimalarial antibody detection according to instruction manuals.

	A	B	C	D	E	Research-based
Antigenic targets	Four recombinant antigens for four species	Recombinant antigens for four species	Recombinant antigens for all *P.* species	Recombinant antigens for four species	CSP and MSP1 proteins from *Pf* and *Pv*	Five *Pf* recombinant antigens*
Subclasses	IgG, IgM, IgA	N/A	IgG, IgM, IgA	N/A	IgG, IgM	IgG
Samples/plate	91	91	89	92	91	80
Duration	90 min	90 min	150 min	135 min	105 min	~48 hours
Plates/run	4	4	4	4	4	40
Specificity according to manual - all species	96% (n=13608)	100% (n=450)	>98%	100%	98%	N/A
Sensitivity according to manual - all species	N/A	98% (n=528)	>95%	94%	96%	N/A
Sensitivity according to manual - *Pf* only	93% (n=76)	98% (n=410)	N/A	N/A	N/A	N/A

For antigen abbreviation see **Chapter 5**, Table 1. Ig: immunoglobulin, N/A: not applicable, min: minutes, *Pf*: *Plasmodium falciparum*.

* AMA-1, MSP1-19, MSP 2 Dd2, MSP2 CH150/9 and GLURP-R2.

Table 2: Costs per sample, amount of serum needed to run a sample, ease-of-use, specificity and cross-reactivity for five commercial (A-E) and the research-based enzyme-linked immunosorbent assays for antimalarial antibody detection.

	A	B	C	D	E	Research-based
Costs/sample [a]	£1.30	£1.32	£1.84	£2.09	£1.71	£0.13
Amount of sample	50 µL	50 µL	150 µL	2 µL	10 µL	2 µL
Ease-of-use [b]	High	High	Low	Medium	Medium	Medium
Proportion negative if:						
Malaria unexposed (n=223)	99%	99%	98%	82%	98%	N/A
***Toxoplasma*-infected, non-exposed** (n=191)	100%	99%	100%	84%	98%	N/A

[a] Commercial assays were bought in bulk (i.e. 25 plates per brand) in January 2016 for Phase I and March 2017 for Phase II. Costs shown here are based on the most recent prices from 2017 for assay A, B and E.
[b] Ease-of-use was assessed based on the number of incubation steps, incubation time, need for sample preparation and whether reagents are ready-to-use (summarised in Supplementary Table 1).
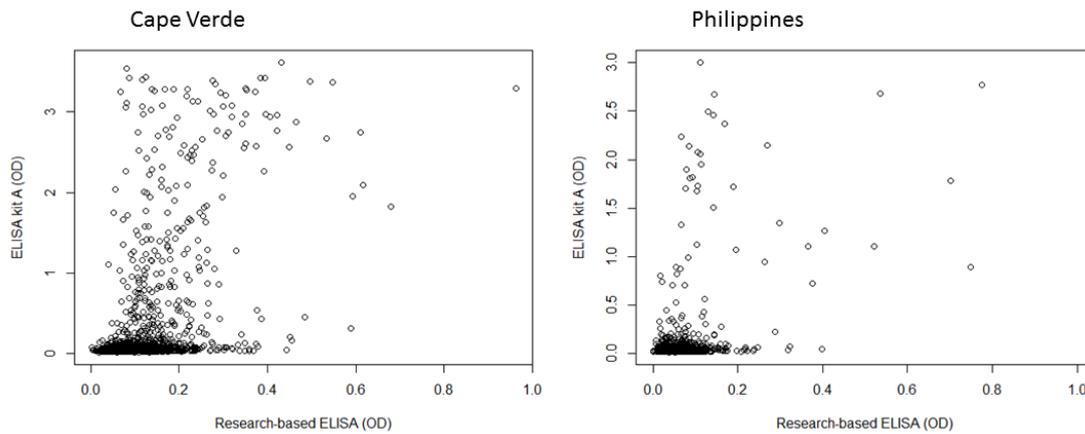
Figure 1: Paired optical density (OD) measurements using the research-based enzyme-linked immunosorbent assay (ELISA) protocol and commercial ELISA kit A in participant samples from Praia, Cape Verde (left) and Bataan, the Philippines (right). OD measures were corrected for blank responses. Open dots represent participant samples while black lines represent thresholds for seropositivity.
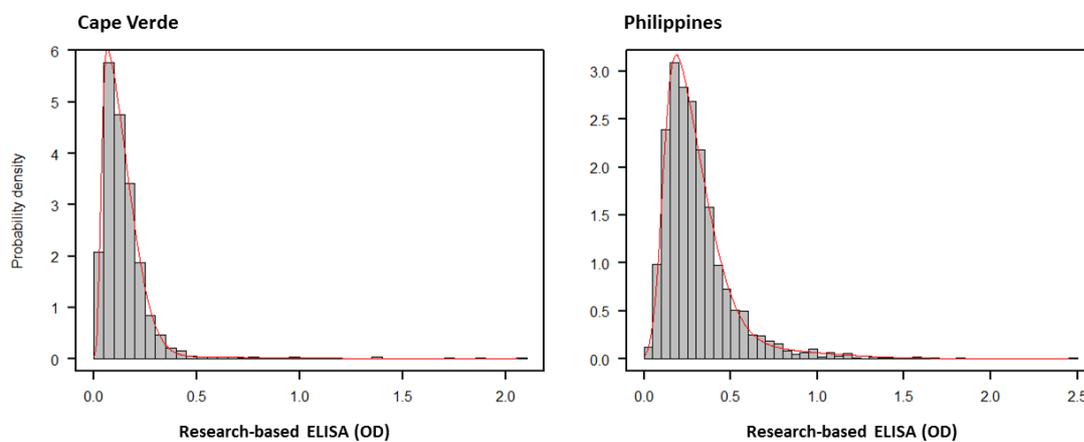
Figure 2: Density plots of optical density (OD) measurements using the research-based enzyme-linked immunosorbent assay (ELISA) protocol in participant samples from Praia, Cape Verde (left) and Bataan, the Philippines (right). OD measures were corrected for blank responses. Red lines represent estimated densities of mixtures of skewed normal distributions. In Cape Verde and the Philippines two mixtures were estimated, however both distributions were assumed be seronegative in Cape Verde (i.e. mean OD_{corr} values for these distributions were 0.04 and 0.14) and the Philippines (0.105 and 0.293).

Supplementary Table 1: Standard operating procedures for five commercial (A-E) and the research-based enzyme-linked immunosorbent assays for antimalarial antibody detection.

	A	B	C	D	E	Research-based
Sample volume (dilution)	50 µl (neat)	50 µl (neat)	150 µl (3:4)	2 µl (1:100)	10 µl (1:101)	2 µl (1:1000)
Incubation	30 (37°C)	30 (37°C)	60 (37°C)	60 (RT)	60 (37°C)	Overnight (4°C)
Wash 1	5	5	4-5	4	3	5
Add conjugate	50 µL (prepare)	50 µL	#1: 150 µL (prepare) #2: 100 µL	100 µL (prepare)	100 µL	1:15,000 (prepare)
Incubation	30 (37°C)	30 (37°C)	#1: 30 + #2: 30 (37°C)	60 (RT)	30 (RT)	180 (RT)
Wash 2	5	5	4-5	4	3	5
Add substrate	50 µL	50 µL	200 µL	100 µL (prepare)	100 µL	100 µL
Incubation	30 (RT)	30 (RT)	30 (RT)	15 (RT)	15 (RT)	15 (RT)
Add stop	50 µL	50 µL	100 µL	50 µL	100 µL	50 µL
Read plate	450nm (reference 630nm)	450nm (reference 630nm)	450nm (reference 630nm)	450nm (blank on air)	450nm (reference 630nm)	450nm

Chapter 7: Discussion

Summary of findings

The overall aim of this thesis was to assess the performance of antimalarial antibody metrics for active detection (i.e. cross-sectional populations) in low transmission and pre-elimination settings. Part of this is a better understanding of the performance of existing malaria metrics at low transmission to identify if/where adjunct metrics are needed. Therefore, **Chapter 3** discussed the discordance between RDT, PCR and microscopy prevalence estimates. It showed that cross-sectional *P. falciparum* parasite rate estimates by microscopy and RDT were similar (microscopy detects 87%, 74–102% of RDT-positive infections), while RDTs miss 41% (26–66%) of PCR-confirmed infections. These observations are consistent with previous observations in a meta-analysis comparing microscopy and PCR prevalence measures [1,2]. These RDT- and/or microscopy-undetected infections were defined in this thesis as low-density infections. The proportion of low-density infections increased with age and decreasing transmission intensity. This suggests that a substantial proportion of malaria infections remain undetected by routine diagnostics in low transmission settings and adjunct metrics are desirable if/where low-density infections need to be identified (discussed below).

Chapter 4 aimed to determine if low-density infections induced measurable antibody responses (IgG). It showed that detectable antibody responses were determined in 96% of previously naïve CHMI participants one month after exposure to low-density *P. falciparum* infections. It should be noted that this was a limited dataset in terms of sample size, genetic diversity of humans (i.e. only from non-endemic settings) and the range of parasite densities acquired. However, it is promising that nearly all participants showed measurable IgG responses one to seven months post-CHMI (≥94% of the participants) while peak parasite densities remained below 200 parasites/µl; and that antibody responses were detected to a small subset of targets (4 out of 40 analysed) in all participants with measurable antibody responses. Even at the low range of peak parasite densities recorded, there was a strong dose-response relationship between cumulative parasite density and MSP-1$_{19}$ as well as a combined measure of the four most responsive antibodies. Thus, a combination of a few antibody responses would have been sufficient in identifying recent exposure to low-density infections in these participants, and, possibly, the individuals with low-density infections in **Chapter 3**.

In commercial ELISA kits, antimalarial antibodies are assessed to a combination of antigenic targets by coating wells with a pool of multiple antigens. Responses to individual targets could not be assessed but results represent a more general estimate of previous exposure to malaria infection, usually used to assess the risk of transfusion-transmitted malaria in donor blood products. In **Chapter 6**, one of five

commercial ELISA kits evaluated was considered applicable for large-scale screening of serum samples in epidemiological surveys and antibody responses recorded with this kit accurately reflected transmission patterns in a low transmission and pre-elimination setting. The use of this standardised serological tool may help as an adjunct measure in supporting claims of the cessation of malaria transmission in previously endemic areas.

Technical considerations

Antibody responses were detected by microarray in **Chapter 4** and ELISA in **Chapter 6**. Multiplex methods, such as protein microarray, have the advantage of screening for multiple antibodies in a sample simultaneously compared to responses to single targets (or a pool) in ELISA. Multiplex bead assays (MBA), another platform for multiplex detection, are increasingly used in malaria research owing to their low costs in acquisition compared to protein microarray (**Chapter 1**). A standardised assay is a pre-requisite for its use in surveillance. However, since the MBA is relatively new to the malaria research field (i.e. first applied to *P. falciparum* antibody detection in 2006 [3]), various methods for normalisation of collected data have been described to standardise results [4,5]. In Haiti, an MBA was used for antibody detection in large-scale malaria transmission surveys which aimed to directly inform control and elimination policies (discussed below). As such, a protocol was designed with an increased throughput: the OneStep protocol in which participant or control/standard serum samples (human IgG) and secondary antibody (anti-human IgG) were incubated simultaneously (Rogier et al. *in preparation*, **Appendix A**). **Chapter 5** contained a discussion of technical considerations for antimalarial antibody detection using the OneStep MBA protocol. Antibody measures in participant samples were shown to increase for all, and saturate for some, antigens if the OneStep protocol was used as compared to a conventional protocol. A statistical approach was applied to adjust for this discordance in antibody measures for participant samples. In addition, **Chapter 5** described the retrospective quality control of data collected with the OneStep protocol in three large-scale malaria transmission surveys by assessing assay precision over time. Inter-plate variability of antibody measures in hyperimmune positive control samples repeated on each plate (i.e. representing assay precision) was considered acceptable. However, inter-plate variability seemed to increase over time, i.e. by the third survey, possibly due to long-term storage of reagents.

Low-density infections: do they matter?

Since the development of RDTs in the 1990s (reviewed in[6]) they have seen improvements in ease-of-use [7] and accuracy [8] and have seen wide-spread use as a routine diagnostic in patient care [9] as well as epidemiological research [10]. For *P. falciparum*, species-specific RDTs detecting circulating HRP2 antigen are most widely used, while LDH-based tests are genus-specific [7]. RDTs are considered user-friendly and fast [7], though they have been designed to detect parasite densities greater than 200 parasites/µl [11]. We now know that most infections exist below this lower limit of detection, especially at low transmission (**Chapter 3**). As the number of countries experiencing low transmission or in pre-elimination increases [12] with our ability to detect lower parasite densities by using ultra-sensitive methodologies involving high blood volumes [13], the question on which limit of detection is needed to eliminate rises. In addition, the time in which identification of these infections can be achieved and therefore the finances needed, need to be considered. Alere™ have developed a highly-sensitive RDT (hs-RDT) with a reported limit-of-detection that is 10-fold more sensitive than conventional HRP2-based RDTs (cRDT). Laboratory assessments of its performance are promising, however results from a limited number of studies to date following field deployment vary [14]. Moreover, reports of parasites with HRP2/HRP3 deletions [15,16] indicate caution as their spread would cause a major public health problem owing to the wide-spread use of HRP2-detecting RDTs [17,18]. Nevertheless, if these deletions are monitored and field deployment of hs-RDTs shows results consistent to laboratory assessments, the use of hs-RDTs may improve the identification of previously undetected populations.

Determining the contribution of low-density to onwards transmission and therefore the answer to the question "*Do they matter?*" was not the aim of this thesis but it is an important part of future work. Some have hypothesised/argued their importance [19]: 1) low-density infections can increase in density at a later timepoint and therefore should be treated [20], 2) although countries have eliminated without attempts to test and treat low-density infections, these may not be representative of remaining endemic settings, and 3) studies aiming to test-and-treat with conventional diagnostics have failed to reduce transmission. Data from observational studies in which human-to-mosquito infectiousness is measured experimentally (i.e. by feeding colony-reared mosquitoes on humans or human blood) is limited, especially due to challenges in finding low-density infections at low transmission (i.e. proportionally they make up the majority of infections, but absolute numbers are low). In Thailand, approximately one in five infections with submicroscopic gametocyte densities for either *P. falciparum* or *P. vivax* were able to infect mosquitoes [21]. Modelling exercises suggest that by increasing the limit-of-detection of RDTs from 200 to 20 parasites/µl, the detected proportion of

the infectious reservoir (i.e. the combined infectivity to mosquitoes of the whole population weighted by how often each individual is bitten) would increase from 55% to 83% [22]. This was based on data collected in Burkina Faso and parameter estimates from Wu et al. (**Chapter 3**). It should be noted that routinely identifying low-density infections might not be needed as MDA strategies have proven successful in decreasing malaria transmission in certain settings [23]. Nevertheless, it is important to determine the contribution of low-density infections (and whether there is a difference in symptomatic or asymptomatic patients) to infectivity of mosquitoes, and thus onwards transmission, in order to design future control and elimination policies. Although PCR-based techniques detect these infections, they are considered impractical for field surveys due to the high costs, long processing time and the lack of appropriate facilities in many endemic countries [24]. Antibody responses may be an option in identifying those exposed to (low-density) malaria infections, however, we need to create a better understanding of the kinetics of antibody responses, their consistency across populations and the limitations or methods of standardisation of antibody detection platforms.

Multiplex antibody detection: less is more

Taking high-throughput to its limits

The application of multiplex antibody detection assays to malaria research has increased the serological information collected from one experiment while decreasing technician time and the quantity of reagents needed. In this thesis, two of these techniques, protein microarray and MBA, are discussed. As mentioned above, MBA is increasingly applied by malaria research groups owing to their low cost of acquisition and ease-of-use compared to protein microarray. Several have described optimisation methods for the MBA such as the detection of Ig subclasses [25] and IgG isotypes [25,26] as well as the optimisation of control standards of hyperimmune sera [25] and analytical methods to assess inter-plate variation using these types of standards [4,5]. Further simplifying the MBA protocol to increase throughput, and decrease technician time and the chance of errors, has also been described (Rogier et al., *in preparation*, **Appendix A**). This is of specific use in settings where results need to be readily available. In the Malaria Zero project in Haiti, serological results were needed rapidly to directly inform control and elimination policies. Antimalarial antibody metrics from rapid assessment surveys were used to stratify areas for MDA (see below) and will be used to assess its impact on malaria transmission in these areas.

A simplified MBA protocol was used for the Malaria Zero surveys in Haiti: the recently described OneStep protocol in which sample (human IgG) and secondary (anti-human IgG) are incubated

simultaneously (Rogier et al., *in preparation*, **Appendix A**). The OneStep MBA protocol involves two wash steps compared to 4-5 in conventional protocols (Rogier et al., *in preparation*, **Appendix A**). This improves the ease-of-use of the assay and limits the chance of errors (e.g. loss of beads through washing steps). The OneStep protocol was also considered to have a higher throughput compared to the conventional protocol. However, the information in the data collected is limited as antibody measures are increased which led to a saturation of MFI measurements at the upper limit-of-detection of the MAGPIX® machine (**Chapter 5**). The saturation in MFI measurements in participant samples was more pronounced for immunogenic targets, as expected, while less immunogenic targets did not reach saturation (**Chapter 5** and **Appendix B**). Assessing antibody responses to a panel of antigens simultaneously means that assay conditions are not optimised individually and thus likely not ideal for all. Even though higher responses were recorded by the OneStep compared to the conventional protocol, seroprevalence estimates were largely the same for malarial antigens (Rogier et al., *in preparation*, **Appendix A**). If the aim of the antibody data collected is stratification of study areas, binary measures may be sufficient, especially combined with age as it creates SCR estimates which have shown a strong correlation with transmission as determined by EIR (**Chapter 1**, [27]). However, if the aim is to inform the effect of policies, seropositivity may not be sufficient, as antibody levels decline prior to changes in seroprevalence [28]. The saturation seen at the higher range of MFI measurements using the OneStep protocol may limit the ability to detect a decrease in antibody titres after the implementation of interventions, especially if pre- and post-intervention surveys are close together in time (i.e. months to a year).

Although the work presented in this thesis has discussed a statistical approach to adjust for the discordance in antibody measures between protocols for participant samples (**Chapter 5**); the data to support this approach was collected at the start of serological data collection for Survey 1. The precision of the assay, assessed by MFI measurements in repeated samples on each plate, decreased over time for some antigenic targets (in Survey 3). Therefore, the use of this statistical approach to transform antibody responses may not be advisable for certain antigens from Survey 3 onwards. Moreover, during my PhD work I have found that, practically, the limiting factor in the time needed to collect data using the MBA lies in reading plates on the MAGPIX® machine rather than in sample processing. Thus, in my opinion, the OneStep protocol does not increase the throughput of samples assayed. Nevertheless, there may be applications of this protocol if 1) the aim is to collect data on seropositivity alone and 2) where results need to be readily available to directly inform control or elimination policies, or for stratification of risk.

Translating results into an actionable response

Commercial ELISAs have been standardised for diagnostic use as well as some research-based protocols. An example is the ELISA protocol used within our research group, which has been used in a variety of endemic settings for almost 15 years [29]. The selected antigens used to coat commercially available ELISAs that detect antimalarial antibodies are unknown for most kits. Some indicate the use of MSP1 and CSP. Based on the results from Bataan, the Philippines, discussed in **Chapter 6**, the antigens used in kit A likely induce antibody responses with relatively long half-lives as high seroprevalence was recorded in adults under the absence of local transmission in the past decades. A research-based ELISA protocol detecting antibodies to a pool of five antigens (including AMA1 and MSP1-19) showed similar though overall lower patterns of age-specific seroprevalence (**Chapter 6, Additional File**). These antibody responses are suitable in describing the cessation of transmission in certain contexts (e.g. in Bataan as age-specific seroconversion curves mirrored a drop from over 800 to 141 reported cases in 1994-1995 after which cases were almost exclusively imported) but likely not all. If the cessation of transmission is more recent, age-specific seroconversion curves may not detect it as shown by relatively high estimates of the recent SCR in Sri Lanka pre-elimination [30]. This could be due to both technical (i.e. assay-specific) or analytical (e.g. sample size/power [31]) issues. Antibody responses with shorter half-lives, as described by Helb et al. [32], may be better suited to describe (the absence of) recent transmission in these settings. Ideally multiplex antibody detection assays would not be used to assess responses to a wide range of malarial targets if the aim is sero-surveillance, owing to the difficulty in translating these data into actionable responses to date. A subset of targets, which, in singular or combined, can represent recent, intermediate and historical exposure, would be advantageous.

It should be noted that there is limited evidence to date on antigens that are associated with recent exposure (i.e. over the past month to one year; Table 1), and even less for those associated with intermediate exposure (i.e. over the past 1-5 or 1-10 years). This information is essential if the aim is to be able to identify different time windows of past exposure to infection in one cross-section as suggested by Greenhouse et al. [27]. Helb et al. identified responses to three antigens that accurately identified whether an individual had been infected within the last 30-365 days (Hyp2, GexP and exonuclease), while continuous responses to a combination of six antigens accurately estimated an individual's malaria incidence in the prior year [32]. However, this cohort was Ugandan children and it is unclear if the same performance would have been observed in adults. Recently described data from a cohort in the Gambia [33], which also included adults, showed some overlap in the antigens associated with recent exposure as identified by Helb at al. but the accuracy was lower, and the identified antigens were different between individuals of all ages and children. Studies from Kenya

and Cambodia also identified antigens associated with recent exposure [34,35]. None of these studies used the same combination of 1) antigen panel assessed, 2) antibody detection assay, 3) sample dilution, or 4) age range of the study population, thus making it difficult to draw conclusions from these results (Table 1). Moreover, it is likely that genetic differences (in human and parasite populations) across endemic settings would induce different immune responses (discussed to some extent in **Chapter 1**) thus more cohort studies are needed to confirm these results.

Once the optimal target(s) are determined for historical, intermediate and recent exposure to malaria infection, these can be tailored to a specific use-case scenario and setting. A single target or a combination of targets can be assessed using a multiplex assay, (pooled) ELISA or LFA[15] depending on the context. The latter two options would be relevant where results have to be readily available (e.g. stratification of areas of risk or a decentralised immediate response). These would also be advantageous for malaria control programme managers, as binary responses (i.e. yes/no) are easier to interpret and respond to than multiple continuous measures. If separate responses to a panel of targets is better suited for a specific use-case-scenario (e.g. measuring the impact of interventions), and therefore a multiplex assay is the most suitable option, the goal should be to expand the panel with antigens from other pathogens of interest in the setting, such as vaccine-preventable diseases, helminths or other parasitic infections to maximise the use of this platform. An overview of the most appropriate serological endpoints using antimalarial antibody metrics per use-case-scenario is shown in **Figure 1**.

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[15] We have tested a newly developed LFA detecting a malaria-related antibody in several low transmission areas, but data were not included due to prototype and patent issues.

Table 1: Overview of published evidence of antibody responses to antigens associated with recent naturally acquired *Plasmodium falciparum* infections.

NB only antigens that are part of the panel analysed in this thesis (Chapter 5-6) are included in this table. Furthermore, only studies with repeated sampling of the same individuals were considered. Recent is defined as ≤1 year.

Description	Target (design)	Detection assay	Sample dilution	Antigens/peptide		Country	Study design	Age range	Ref
Accurately predicted day since last infection (30, 90 or 365 days)[a]	Recombinant antigens (IVTT)	Micro-array	1:200	Hyp2 GexP	HSP40 Etramp 4	Uganda	Cohort	3-7 years	[32]
Predicted incidence (symptomatic malaria) in previous year	Recombinant antigens (IVTT)	Micro-array	1:200	Hyp2 Etramp 5 CSP	MSP2 Etramp 4	Uganda	Cohort	3-7 years	[32]
Accurately predicted day since last infection (30, 90 or 150 days)[b]	Recombinant antigens (bespoke)	MBA	1:400	GexP HSP40	Etramp 5 AMA1	The Gambia	Cohort	All ages	[33]
Accurately predicted day since last infection (30, 90 or 150 days)[c]	Recombinant antigens (bespoke)	MBA	1:400	SBP1 GexP Rh5 Etramp 5 EBA175	HSP40 AMA1 MSP1-19 GLURP	The Gambia	Cohort	1-15 years	[33]
Estimated half-life <1 year[d]	Peptides and recombinant antigens (bespoke)	MBA	1:200	GLURP-R2 MSP1-19	CSP (peptide)	Cambodia	Re-sampled population from repeated cross-sectional surveys	2-50 years	[34]
Estimated half-life <1 year[e]	Recombinant antigens (bespoke)	MBA	1:200	CSP (peptide)		Kenya	Cohort	All ages	[35]
Estimated half-life <1 year[e]	Recombinant antigens (bespoke)	MBA	1:200	AMA-1 EBA175	MSP1-19 GLURP-R2	Kenya	Cohort	<5 or <10	[35]

[a] Area under the curve (AUC) of ROC > 0.85. [b] AUC of ROC ≥ 0.78. [c] AUC of ROC ≥ 0.79. [d] Estimations based on antibody loss over ~600 days (~20 months). [e] Estimations based on antibody loss over ~14 months. IVTT: in vitro transcription and translation; MBA: multiplex bead assay
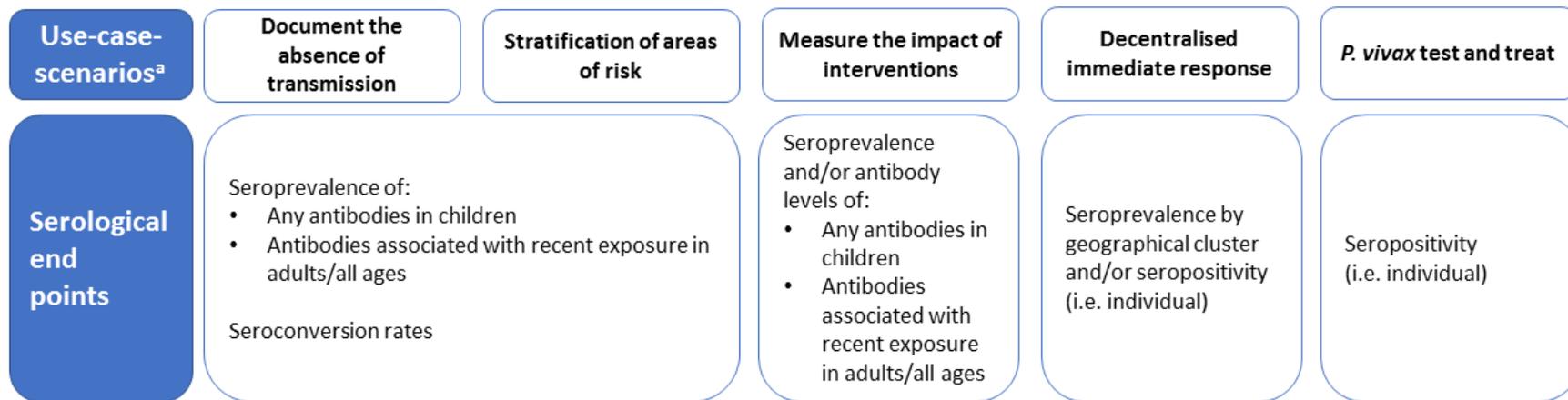
Figure 1: Previously described use-case-scenarios of antimalarial antibody detection and hypothesised optimal serological endpoints using antimalarial antibody metrics. [a] Use-case -scenarios were described by Greenhouse et al. (*under review*, Gates Open Research).

Multiplexing across diseases rather than within

Arnold et al. recently advocated for "an integrated approach to surveillance of population immunity and infectious disease transmission" [36]. They highlighted that in the last 10 years Uganda has completed 20 or more population-based sero-surveillance surveys across multiple infectious diseases. Integrating these serological surveys can greatly reduce costs and time. MBA protocols require as little as 1 μl of serum to determine up to 50 or 100 antibody responses simultaneously. This means that collection of participant samples could be reduced by expanding antigen panels to include more pathogens, if study designs are appropriate for the pathogen of interest. This would greatly reduce survey and laboratory costs but may also help in ensuring continued community participation as repeated sampling of the same populations may lead to reduced participation (reviewed in [37]), especially as transmission decreases and perceptions about personal and/or perceived risks change [38]. Moreover, an integrated approach in collecting serological data may also engage scientists in exchanging statistical approaches to determine disease transmission and changes therein. Currently, MBA platforms seem to mostly be applied to assess multiple antibodies within one disease, such as in the Malaria Zero project in Haiti, while future work should focus on including antigens from other pathogens to make optimal use of the information gathered in cross-sectional surveys.

In addition to multiplex antibody detection, MBA platforms have also been used to detect human polymorphisms to identify inherited blood disorders associated with malaria [39] or *Plasmodium* polymorphisms to identify sensitivity to antimalarial treatment [40]. Furthermore, protocols have been described to detect parasite antigen in samples such as HRP2 and pLDH to validate RDT survey results [41], thereby making it possible to screen for infections with possible HRP2/HRP3 deletions more efficiently [42]. Creating and/or optimising these protocols to include the detection of other pathogens would further integrate surveillance methods.

Limitations

PhD project-specific issues

The order in which the projects are presented in this thesis does not represent the chronological order in which they occurred. Ideally, the results from the CHMI project (**Chapter 4**), and/or other longitudinal studies conducted within our and other research groups, would have informed the selection of the antigen pool tested by ELISA in **Chapter 6** (**Additional File**). However, the commercial ELISA project preceded the CHMI project, thus a pool of well-characterised antibodies with relatively long half-lives was used. The pooled, research-based ELISA protocol would be ideal for rapid

assessment of population exposure history due to its ease-of-use and relatively low cost per sample. Moreover, national laboratories in endemic settings generally have access to a spectrophotometer due to its application in monitoring or diagnosing other infectious diseases such as Human Immunodeficiency Virus (HIV). Future work to optimise the research-based ELISA protocol for antimalarial antibody detection would include increasing serum concentration as well as blocking non-specific binding to improve discrimination between negative and positive antibody responses. The advantage of the research-based ELISA compared to commercial ELISAs is the fact that antigen pools (i.e. those with long or short half-lives) can be selected for specific use-case-scenarios and settings.

The projects presented in this thesis were not all part of the original outline of chapters. My role in the Malaria Zero project, in which I worked part-time throughout my PhD, included analyses and interpretation of antibody responses from cross-sectional surveys to inform control and elimination strategies. In addition, I focused on a comparison of antibody metrics by survey design: a cross-sectional household survey compared to a survey in easy-access-groups, such as children at schools or visitors of health facilities. A one-year delay in cross-sectional surveys due to hurricane Matthew causing landfall in Haiti in September 2016, as well as programmatic issues, led to the exclusion of these projects from the thesis. Throughout the duration of my PhD I have presented posters at scientific conferences detailing the use of the antibody data collected in Haiti in informing control and elimination policies. These posters are included in **Appendix E** and represent an overview of the statistical approaches I have learned during this time (e.g. STATA and later R Studio). Although not included in this thesis, working with these data and for the Malaria Zero project has helped me advance my understanding of antimalarial antibody metrics at low transmission. It has also given me additional field experience, such as in the setup, deployment and performance of cross-sectional surveys.

Limitations in the use of antimalarial antibodies at low transmission

Outstanding issues in the use of antimalarial antibody metrics were detailed in Figure 4, **Chapter 1**. Some of these were focussed on low transmission and pre-elimination. The performance of antimalarial antibodies in detecting recent exposure to low-density infections has been demonstrated in this thesis (**Chapter 4**), although a limited sample size was available, and the results need to be confirmed following naturally-acquired infections. However, for antibody metrics to be applied to sero-surveillance in low transmission and pre-elimination settings, there are still some unresolved issues.

Firstly, the most accurate standardised method to determine thresholds for seropositivity needs to be established. In low transmission and pre-elimination settings, an approach in which latent distributions are identified in the antibody data (such as finite mixture models) may not be appropriate. Specifically, the decreasing seropositive population may not be sufficient to identify as a separate population and they may instead be identified as outliers of the seronegative population (i.e. false-negatives). Or vice versa, outliers of the seronegative population may be identified as a separate population and incorrectly be identified as seropositive (i.e. false-positives). This may especially affect antibodies with short half-lives which were hypothesised to be able to detect (the absence of) malaria transmission earlier than those with long half-lives. Therefore, alternative methods should be explored to harness the information gained from short-term antibodies reliably. It may instead be more appropriate to identify thresholds using the antibody responses of an unexposed reference seronegative population from the same or similar setting (e.g. those in metropolitan cities of endemic countries where transmission can be lower or even non-existent, or those who have migrated to non-endemic settings).

Secondly, although antibodies with short half-lives representing recent exposure may be advantageous for certain use-case-scenarios compared to those with long half-lives (e.g. to measure the effect of interventions), they may not be ideal at low transmission or pre-elimination. An advantage of antimalarial antibodies metrics is the smaller sample sizes needed to measure transmission as they represent cumulative exposure. However, as transmission declines to less than 1 case per 1000, short-lived antibodies (i.e. <1 year) would arguably require similar sample sizes to those detecting clinical cases , and perhaps even asymptomatic infection, in order to measure transmission.

Conclusion

The data presented in thesis has furthered our understanding of the use of antimalarial antibody metrics to determine malaria transmission at low transmission and pre-elimination. It has concluded that 1) RDT detects about 40% of all PCR-confirmed infections and RDT-undetected infections increased with age and decreasing transmission intensity; 2) low-density infections in previously naïve CHMI participants induced measurable IgG responses and antibodies to only four targets represented all participants with a measurable IgG response; 3) MBA protocols can be optimised for particular use-case-scenarios (rapid screening of large sample numbers); 4) a commercially available, standardised ELISA kit, in which antimalarial antibodies were measured to a pool of antigens, was applicable to large-scale screening of samples in epidemiological surveys, and responses detected by this kit mirrored transmission patterns represented by passively detected case counts in a low transmission

and pre-elimination setting. The work in this thesis has also identified areas of possible future research: 1) to determine the contribution of low-density infections in humans to infectivity of mosquitoes, and therefore onward transmission; 2) to determine which antibodies reflect recent exposure following naturally acquired low-density infections in endemic populations; 3) to improve pooled ELISA protocols by either optimising commercial ELISA kits for the use of lower serum concentrations (i.e. enabling the use of dried blood spots), or by optimising research-based protocols by increasing serum concentrations with more efficient blocking of non-specific binding, pooling markers of recent exposure and/or detecting IgM antibodies.

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Appendices

Appendix A: Sections of manuscript draft: High-Throughput Integrated Disease Serosurveillance using a One-Step Multiplex Bead Assay. Rogier et al. *in preparation*.

Appendix B: Transformation of antibody responses between the OneStep and Stepwise multiplex bead assay protocols for remaining antigenic targets

Appendix C: Instruction Manuals of Commercially Available Enzyme-linked Immunosorbent Assays

Appendix D: Standard Operating Procedure for the Research-based Combined Antigen Enzyme-linked Immunosorbent Assay

Appendix E: Posters Presenting Results from the Malaria Zero Project for Scientific Conferences Visited During my PhD programme

Appendix A: Sections of manuscript draft: High-Throughput Integrated Disease Serosurveillance using a One-Step Multiplex Bead Assay. Rogier et al. *in preparation*.

High-Throughput Integrated Disease Serosurveillance using a One-Step Multiplex Bead Assay

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Methods

Samples. Serum and dried blood spot (DBS) samples (n=796 DBS with 712 paired serum) from serosurvey in Haiti were collected in April and May 2017. Informed consent was collected from all participants enrolled if older than 18 years, and informed consent from a legal guardian if the participant was younger than 18. Fingerprick whole blood was collected in EDTA-coated capillary tubes (Safe-T-Fill™ Capillary Blood Collection Systems: EDTA, # 07 7053, RAM Scientific Inc., Yonkers, NY) and whole blood was spotted on Whatman 903 ProteinSaver cards (GE Healthcare) on the same day. Blood remaining in the tube was stored at 4°C until later centrifugation (5000g for 2min) to fractionate and allow removal of serum. The study protocol was approved by Haiti Ethical Review Committee; CDC investigators were determined not to be engaged with human subjects.

DBS from all studies were eluted in Buffer B (PBS containing 0.5% BSA, 0.05% Tween 20, 0.02% sodium azide, 0.5% polyvinyl alcohol, 0.8% polyvinylpyrrolidone and 0.5% w/v *E. coli* extract) by incubation overnight at 4 degrees. Liquid serum from the Haiti survey was directly diluted in Buffer B.

Antigens and Couplings. All antigens were covalently linked to MagPlex (magnetic) microspheres (Luminex Corp., Austin, TX) as described previously [25]. Briefly, beads were pulse vortexed, transferred to a microcentrifuge tube and centrifuged for 5 minutes at 16,000g. Supernatant was removed and beads were washed with 0.1M sodium phosphate, pH 6.2 (NaP). Beads were activated by suspending in NaP with 50 mg/mL of EDC (1-ethyl-3-[3-dimethylaminutesopropyl] carbodiimide hydrochloride) and 50 mg/mL sulfo-NHS (sulfo N-hydroxylsulfosuccinimide) and incubating with rotation for 20 minutes at room temperature (RT) protected from light. After wash with antigen-coupling buffer (optimized for each antigen), beads were suspended in antigen coupling buffer with the appropriate concentration of antigen and rotated for 2 hours at RT protected from light. Beads were washed and suspended in PBS with 1% bovine serum albumin (BSA) and incubated for 30

minutes at RT by rotation. Beads were then washed with storage buffer (PBS, 1% BSA, 0.02% sodium azide and 0.05% Tween-20) and and suspended in storage buffer containing protease inhibitors (200 µg/mL Pefabloc, 200 µg/ml EDTA, 1 µg/mL pepstatin A and 1 µg/mL leupeptin) and stored at 4°C. Coupled beads were counted with either a hemocytometer or Luna cell counter using manufacturer's instructions (Logos Biosystems Annandale, VA).

Bead-based Immunoassay Protocols. The standard MBA was performed as described previously [25]. Briefly, the standard assay was performed in flat bottom BioPlex Pro 96 well plates (Bio-Rad, Hercules, CA). Washes between incubation steps used a handheld magnet (Luminex Corp). After addition of 200µl wash buffer (PBS, 0.05% Tween-20, PBST) to each well, wash buffer was left in each well for one minute to allow bead magnetization before inverting the plate to evacuate the wells of liquid. Beads (250,000 beads/antigen/plate) were suspended in Buffer A (PBS, 0.5% BSA, 0.05% Tween-20, 0.02% NaN_3) and 50 µL bead mix added to each well. Plates were washed two times with PBST and 50 µL of sample was added to each well and incubated with shaking at room temperature for 90 minutes. After 3 washes with PBST, beads were incubated with biotinylated anti-human IgG (1:500, Southern Biotech, Birmingham, AL) and biotinylated anti-human IgG_4 (1:625, Southern Biotech). Plates were incubated for 45 minutes and washed 3 times with PBST. Streptavidin conjugated to phycoerythrin (PE) (1:200 Invitrogen, Waltham, MA) was added to detect bound secondary antibody. After a 30 minute incubation, wells were washed 3 times with PBST and incubated in Buffer A for 30 minutes under light shaking to remove any loosely bound antibodies. Samples were resuspended in 100µl PBS and fluorescence data collected immediately on the MAGPIX with Bio-Plex Manager™ MP software with a target of 50 beads per region per well. Median fluorescence intensity (MFI) signal was generated for a minimum of 50 beads/region, and background MFI from wells incubated with Buffer B was subtracted from each sample to give a final value of MFI minus background (MFI-bg) for analysis.

The OneStep assay was performed with the same samples and reagents used in the standard MBA protocol. In 5mL Buffer A, a bead mix was prepared with all regions included,

and 50uL bead mix was pipetted into a BioPlex Pro plate. Beads were washed 2x with 100uL PBST, and 50uL reagent mix (in 5mL Buffer A: 1:500 anti-human IgG, 1:625 anti-human IgG_4, 1:200 streptavidin-PE) was added to all wells, then 50µL samples (or controls) were added to the appropriate wells. Plates were incubated overnight with gentle shaking at room temperature and protected from light. The next morning (after ~16h total incubation time), plates were washed 3x, and beads resuspended with 100µL PBS and read on the MAGPIX machine. MFI signal was generated for a minimum of 50 beads/region, and background MFI from wells incubated with Buffer B was subtracted from each sample to give a final value of MFI-bg. After reading, plates were evacuated of sheath fluid, and beads resuspended in 100µL PBS, sealed, and stored at 4°C to allow testing of the same plates at a later date. A flow chart comparing the two protocols is shown in Figure 1.

Statistical Analysis. Statistical analyses were performed in SASv9.4 (SAS Institute, Cary, NC). Direct comparisons between MFI-bg values using the two protocols were represented by *k*-nearest-neighbor-based local regression (LOESS) curves created through the SGPLOT procedure with cubic interpolation and a degree of 2. Log-transformed MFI-bg values were fit to a two-component finite mixture model by the FMM procedure with normal distribution and maximum likelihood estimation outputs. Linear regression was used to estimate change in MFI-bg values following storage for one week or one month after completion of the OneStep protocol.

Results

Selection of Samples and Antigens Used for the Study. To broadly evaluate the impact of modifying the assay protocol on the fluorescence detection signal (Fig. 1), we tested a diverse set of 511 samples from multiple global locations representing an array of different infectious disease settings: United States, Brazil, Ghana, Tanzania, and Mozambique[16]. For

collection of IgG data, a total of 36 antigens were chosen representing 25 pathogens (viral, bacterial, and parasitic) capable of causing disease in humans.

Use of the Finite Mixture Model Statistical Approach to Categorize Dataset. We used the 2-component finite mixture model (FMM) statistical approach to estimate the antibody distributions in two putative subpopulations for each antigen: seropositive and seronegative [17,18]. This statistical approach was chosen as it could be applied to the MFI-bg data for all 36 antigens used in this study, and the maximum likelihood estimation (MLE) outputs could be directly compared between the two protocols.

Comparison of Prevalence Estimates from Integrated Serosurvey in Haiti. To compare seroprevalence estimates that would be generated for defined study population using different laboratory protocols or sample types, we used samples collected during an integrated serosurvey in Haiti. Serum and dried blood spot (DBS) samples from the same persons were assayed by the standard and OneStep protocols for IgG against a select panel of eight antigens representing malaria, strongyloidiasis, and lymphatic filariasis. Similar to the findings from the sample set representing multiple countries, the assay signals generated by using the OneStep protocol to test Haitian samples were amplified for all eight antigens included in the integrated serosurvey regardless of the serum or DBS sample type (Supplementary Fig. 4). In applying the 2-component FMM for the eight antigens, increased distance was seen between the means of the two components for data for 7 of 8 antigens tested in serum, and 7 of 8 antigens tested from DBS (Supplementary Fig. 5, Supplementary Table 3). Estimates for seroprevalence in the Haiti study population were largely unchanged if different sample types or test protocols were used (Table 2). Notable exceptions were the higher seroprevalence estimates (greater than 1.28 standard deviations) observed for use of the OneStep protocol with serum samples for NIE, Bm14, and Bm33NS antigens.

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Tables

Table 2. Seroprevalence Estimates for Percent of Population Positive to IgG against Specified Antigens, Haiti 2017

Antigen	Serum		Dried Blood Spot		Average Estimate (s.d.)
	Standard	OneStep	Standard	OneStep	
Pf MSP1-19	45.7	38.8	46.6	42.4	43.4 (3.54)
Pv MSP1-19	5.8	8.9	7.0	7.9	7.4 (1.32)
Pm MSP1-19	6.2	8.3	6.3	5.9	6.7 (1.10)
PfCSP	16.4	18.8	15.6	13.8	16.2 (2.07)
NIE	3.4	6.2	2.9	1.8	3.6 (1.87)
Wb123	4.5	5.3	2.8	6.7	4.8 (1.63)
Bm14	18.6	29.1	13.8	16.4	19.5 (6.71)
Bm33NS	15.6	42.1	14.1	13.1	21.2 (13.95)

Figures

Standard Protocol   OneStep Protocol
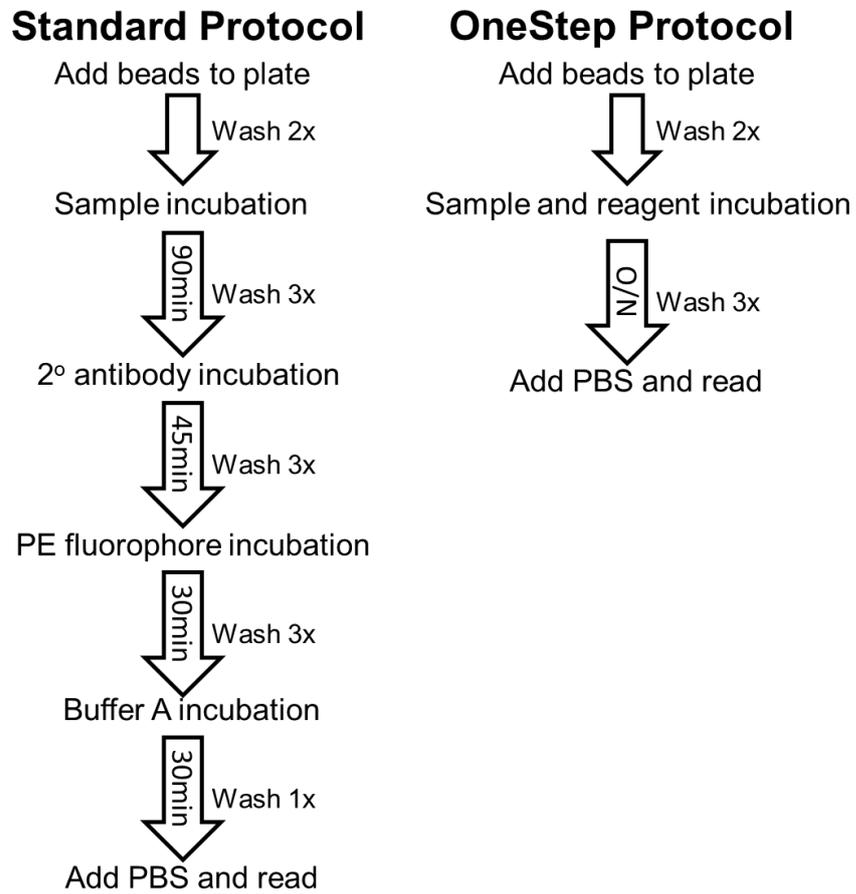
Figure 1. **Protocols for standard and OneStep MBA.** Each step of assay protocols is outlined with time between steps indicated in the arrows.

Supplementary Information

Supplementary Table 3. Estimates and Fitting Statistics from Finite Mixture Model Comparing Standard to OneStep Protocols, and Blood Elutions to Serum, from Haiti Samples

Antigen	Sample Type	Protocol	Component 1 lognormal mean (variance)	Component 2 lognormal mean (variance)	Δ lognormal means C1 vs C2
Pf MSP1-19	Serum	Standard	3.6 (0.50)	8.1 (3.64)	4.5
		OneStep	4.7 (2.65)	10.5 (0.29)	5.8
	Blood Elution	Standard	3.5 (0.25)	7.3 (4.21)	3.8
		OneStep	4.0 (0.71)	10.1 (1.43)	6.1
Pv MSP1-19	Serum	Standard	4.2 (0.25)	5.6 (1.9)	1.4
		OneStep	4.1 (0.45)	6.0 (2.66)	1.9
	Blood Elution	Standard	4.1 (0.19)	5.3 (1.17)	1.2
		OneStep	4.2 (0.25)	5.6 (1.9)	1.4
Pm MSP1-19	Serum	Standard	3.7 (0.26)	5.5 (1.84)	1.8
		OneStep	3.9 (0.35)	6.2 (2.89)	2.3
	Blood Elution	Standard	4.1 (0.18)	5.5 (0.85)	1.4
		OneStep	4.2 (0.27)	6.2 (2.15)	2.0
PfCSP	Serum	Standard	3.7 (0.45)	6.1 (3.28)	2.4
		OneStep	4.1 (0.66)	7.5 (4.14)	3.4
	Blood Elution	Standard	3.5 (0.31)	5.7 (2.85)	2.2
		OneStep	4.1 (0.38)	7.4 (4.41)	3.3
NIE	Serum	Standard	4.0 (0.40)	5.9 (2.53)	1.9
		OneStep	4.4 (0.48)	6.1 (2.87)	1.7
	Blood Elution	Standard	4.3 (0.30)	5.5 (1.30)	1.2
		OneStep	5.1 (0.51)	6.9 (2.62)	1.8
Wb123	Serum	Standard	4.1 (0.22)	5.2 (0.75)	1.1
		OneStep	4.4 (0.36)	5.9 (1.30)	1.5
	Blood Elution	Standard	4.2 (0.10)	4.9 (0.37)	0.7
		OneStep	5.1 (0.30)	7.2 (0.49)	2.1
Bm14	Serum	Standard	3.8 (0.30)	6.3 (0.54)	2.5
		OneStep	4.0 (0.26)	6.6 (1.12)	2.6
	Blood Elution	Standard	3.8 (0.17)	5.7 (1.19)	1.9
		OneStep	4.2 (0.25)	6.1 (1.81)	1.9
Bm33NS	Serum	Standard	5.3 (0.64)	7.4 (1.63)	2.1
		OneStep	4.8 (0.46)	7.8 (2.39)	3.0
	Blood Elution	Standard	5.2 (0.32)	6.9 (1.58)	1.7
		OneStep	5.7 (0.55)	8.8 (1.30)	3.1
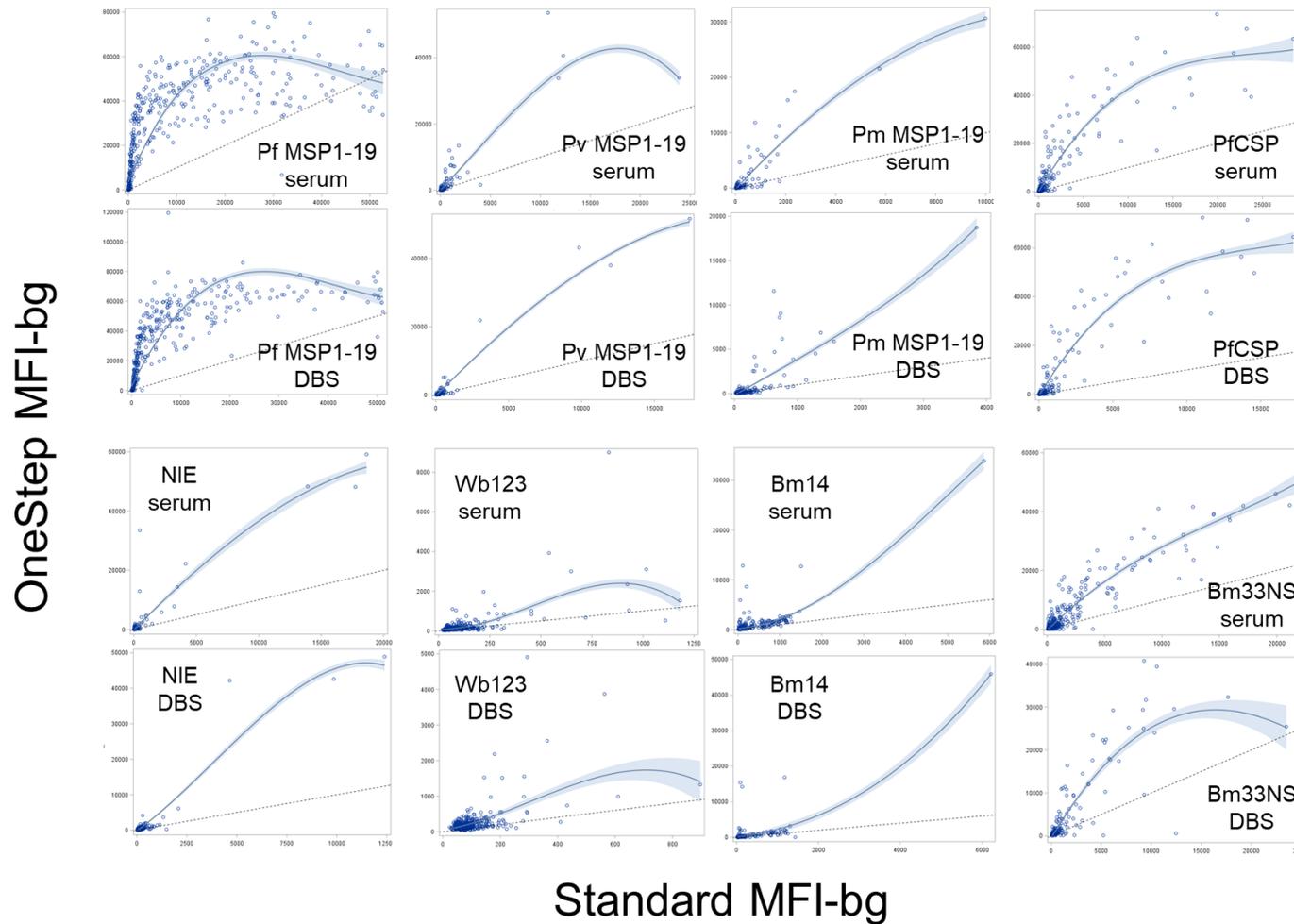
Supplementary Figure 4. Median Fluorescence Intensity Minus Background (MFI-bg) Signal Comparison for Serum and Dried Blood Spot Sample Types from a Serosurvey in Haiti, Assayed Using Standard or OneStep Protocols. Relationship between two protocols visualized as LOESS curves with cubic interpolation and 95% confidence intervals in shading, with y=x reference as a hashed line.
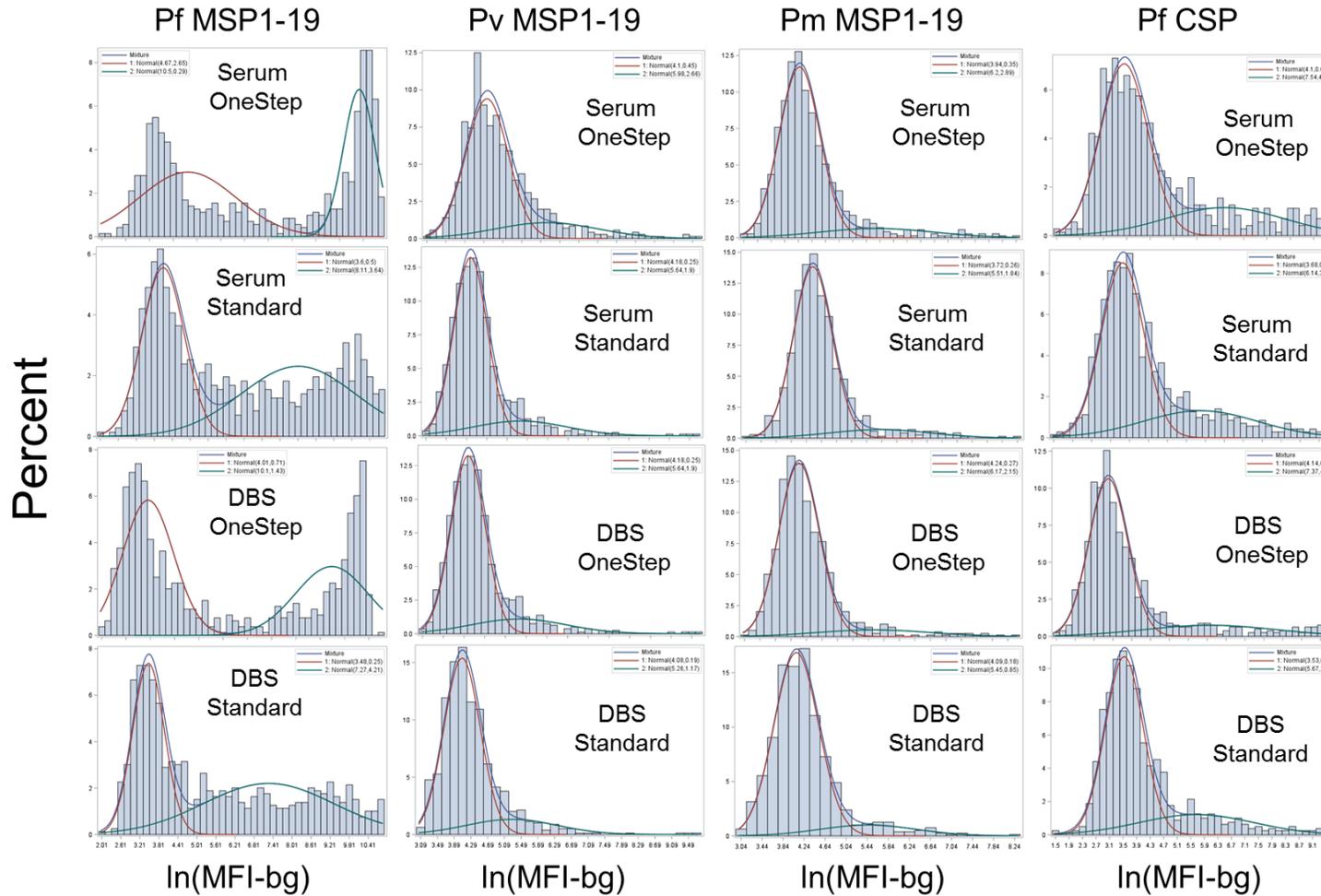
Supplementary Figure 5. Fittings to Two-Component Finite Mixture Models for Antigen Data from Haiti Study Collected by both Assay Protocols for both Serum and Dried Blood Spot Sample Types. Histograms are displayed for log-transformed MFI-bg values for the entire sample set as generated by the standard and OneStep protocols for serum and dried blood spot data with percent of sample set on y-axes.
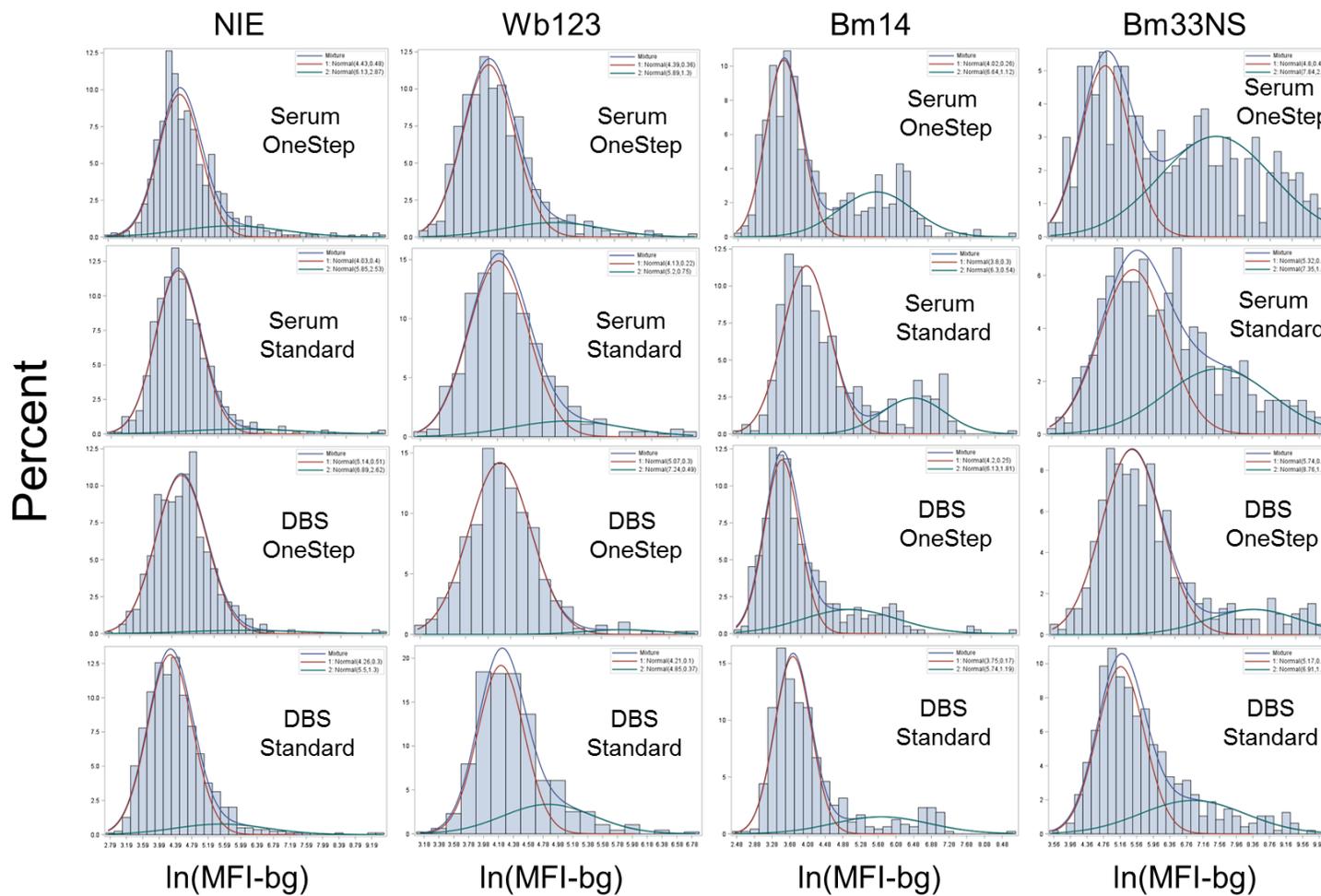
Supplementary Figure 5. Continued.
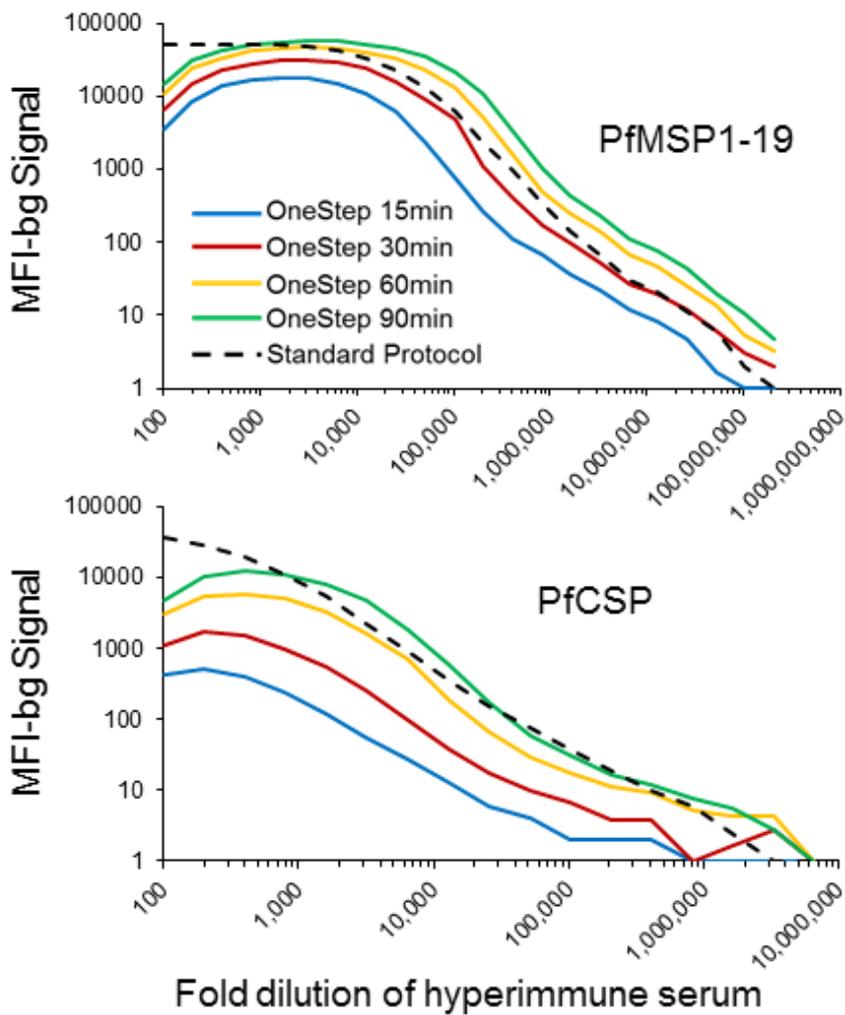
Supplementary Figure 6. Different Incubation Times for the OneStep Assay and MFI-bg Assay Signal for Selected Malaria Antigens. Hyperimmune serum for malaria antigens was serially-diluted and incubated for 15, 30, 60, or 90 minutes with OneStep protocol, or assayed with standard protocol.
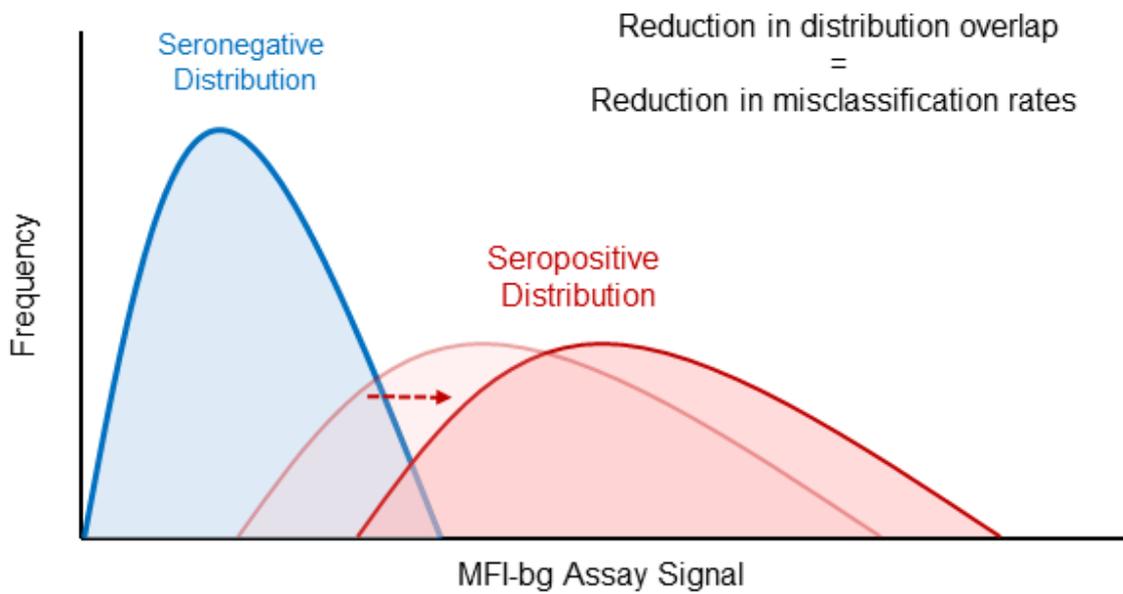
Supplementary Figure 7. Illustration of the Positive Shift in the Assay Signal Distribution for Seropositive Persons as Observed for IgG Data against Most Antigens when Using the OneStep Protocol as Compared to the Standard Protocol. Signal distribution for seronegative persons remained largely unchanged.

Appendix B: Transformation of antibody responses between the OneStep and Stepwise multiplex bead assay protocols for remaining antigenic targets
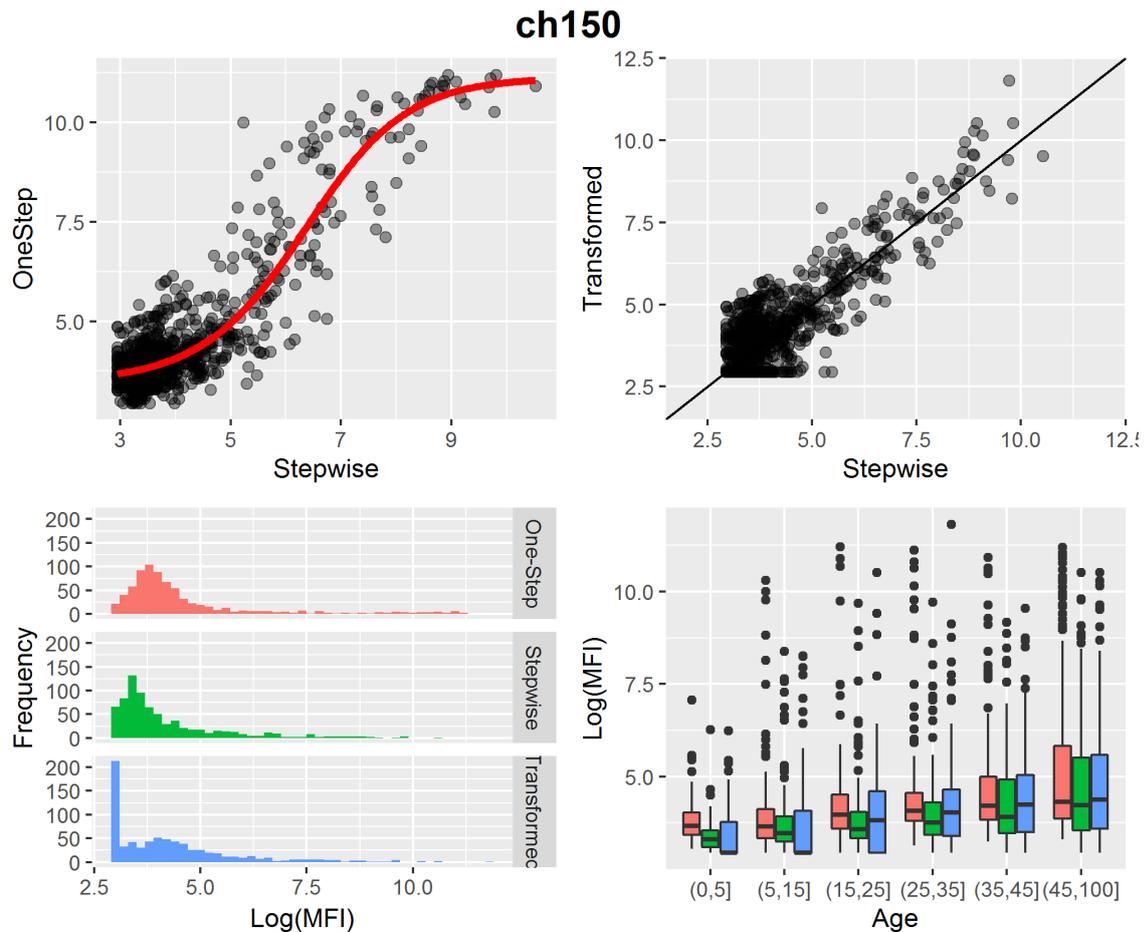
ch150

Transformation of antibody responses between the OneStep and Stepwise multiplex bead assay protocols for MSP2 CH150/9. Median fluorescence intensity (MFI) measurements were corrected for background reactivity of blank responses and natural log-transformed. For antigen abbreviations see **Chapter 5**, Table 1. The sigmoidal relationship of paired measurements was obtained using 804 samples processed on the One-Step and the Stepwise assay protocol (Rogier et al., *in preparation*, **Appendix A**). (**a**) Scatter plot of paired measurements with 4 or 5-parameter logistic regression in red line. Curve parameters of sigmoidal fits are shown in Supplementary Table 2. Recorded MFI measurements lower than blank responses were replaced with the average of blank MFI values of the One-Step protocol across all plates by antigen. Measurements from the One-Step protocol were transformed using the sigmoidal fit. Samples that fell below the bottom asymptote or over the upper asymptote, were replaced by the lowest and highest values that the model could estimate. (**b**) Scatter of transformed responses compared to measurements using the Stepwise protocol. (**c**) Histograms of One-Step, Stepwise and transformed antibody measurements. (**d**) Box plots of age-specific One-Step, Stepwise and transformed antibody measurements.
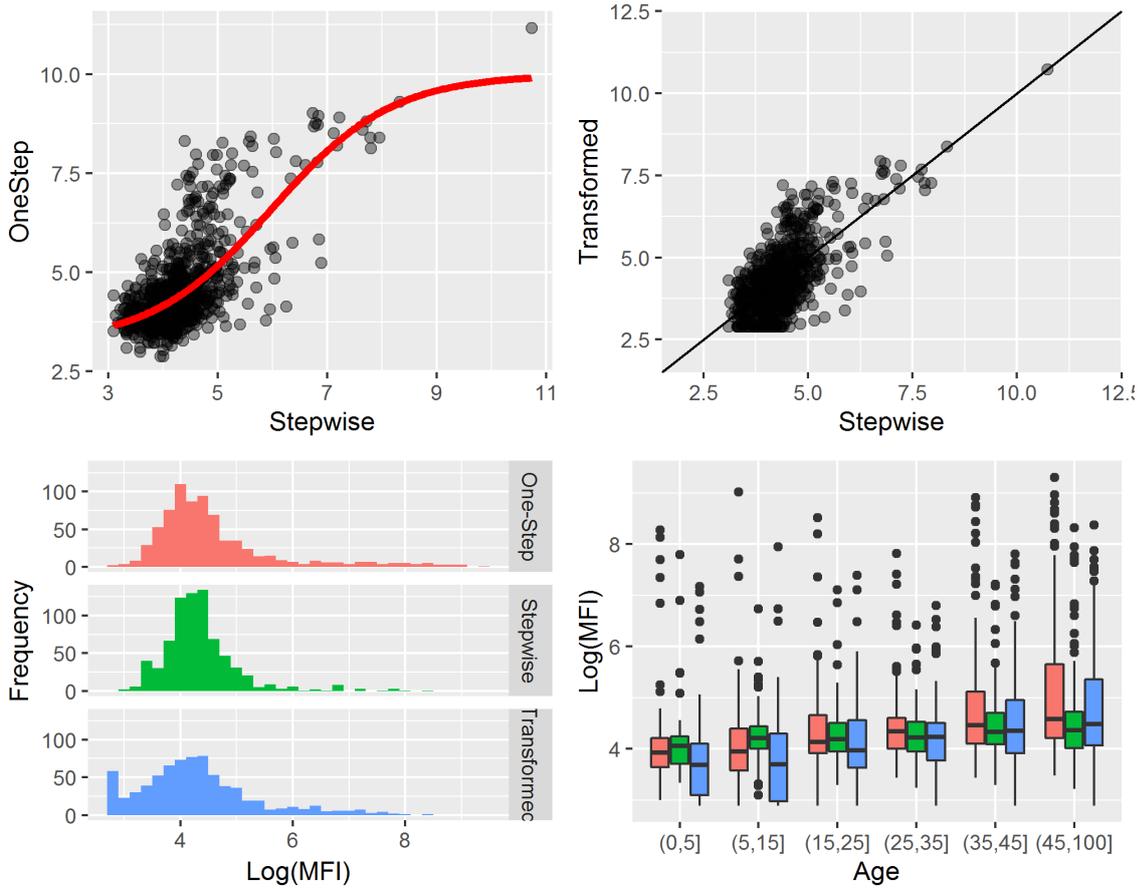
Transformation of antibody responses between the OneStep and Stepwise multiplex bead assay protocols for MSP2 Dd2. For legend see page 218.
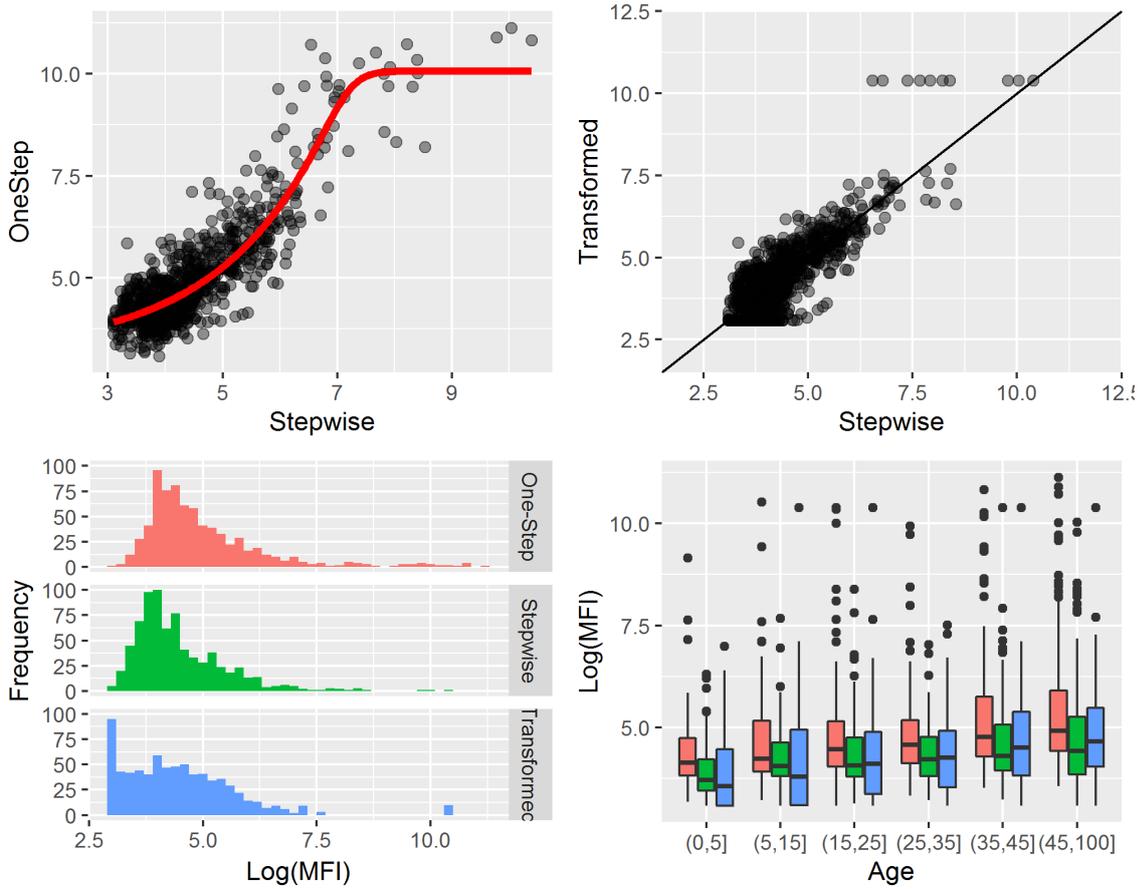
Transformation of antibody responses between the OneStep and Stepwise multiplex bead assay protocols for Etramp 4 Ag 2. For legend see page 218.
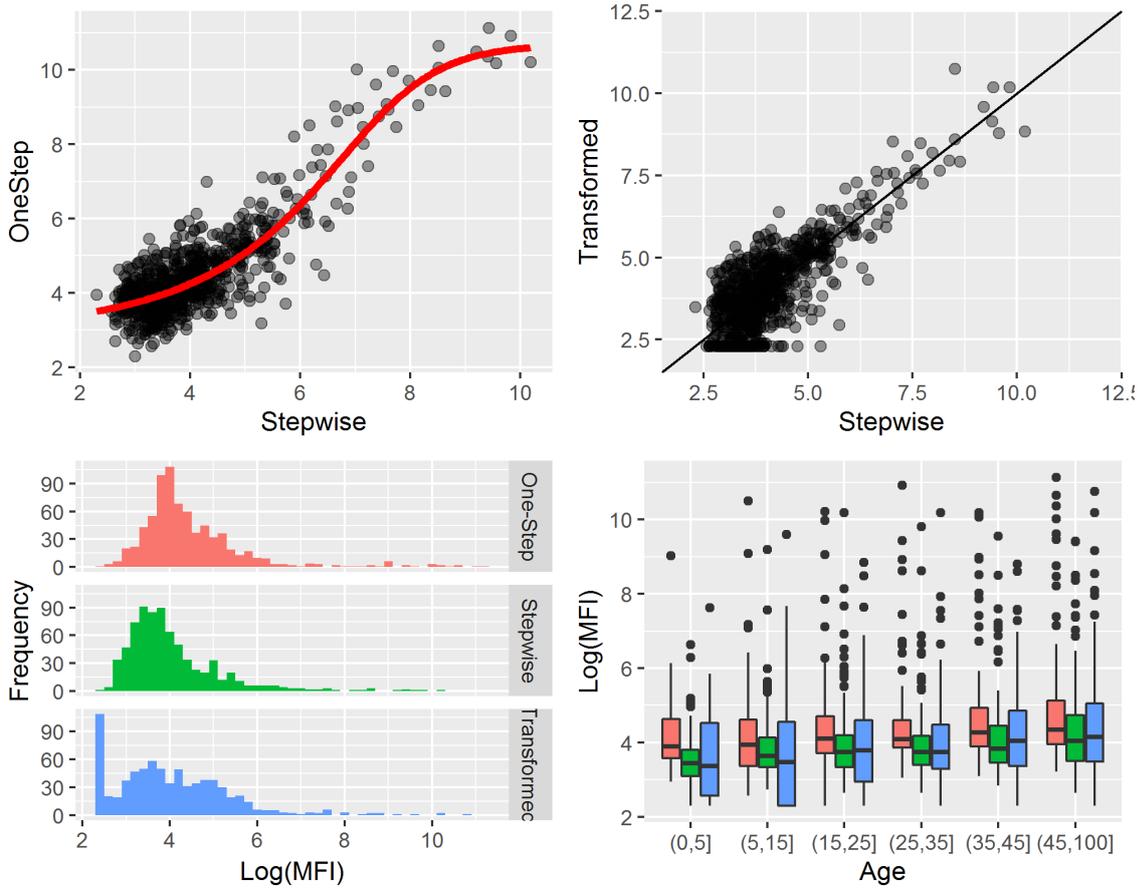
Transformation of antibody responses between the OneStep and Stepwise multiplex bead assay protocols for Etramp 5 Ag 1. For legend see page 218.
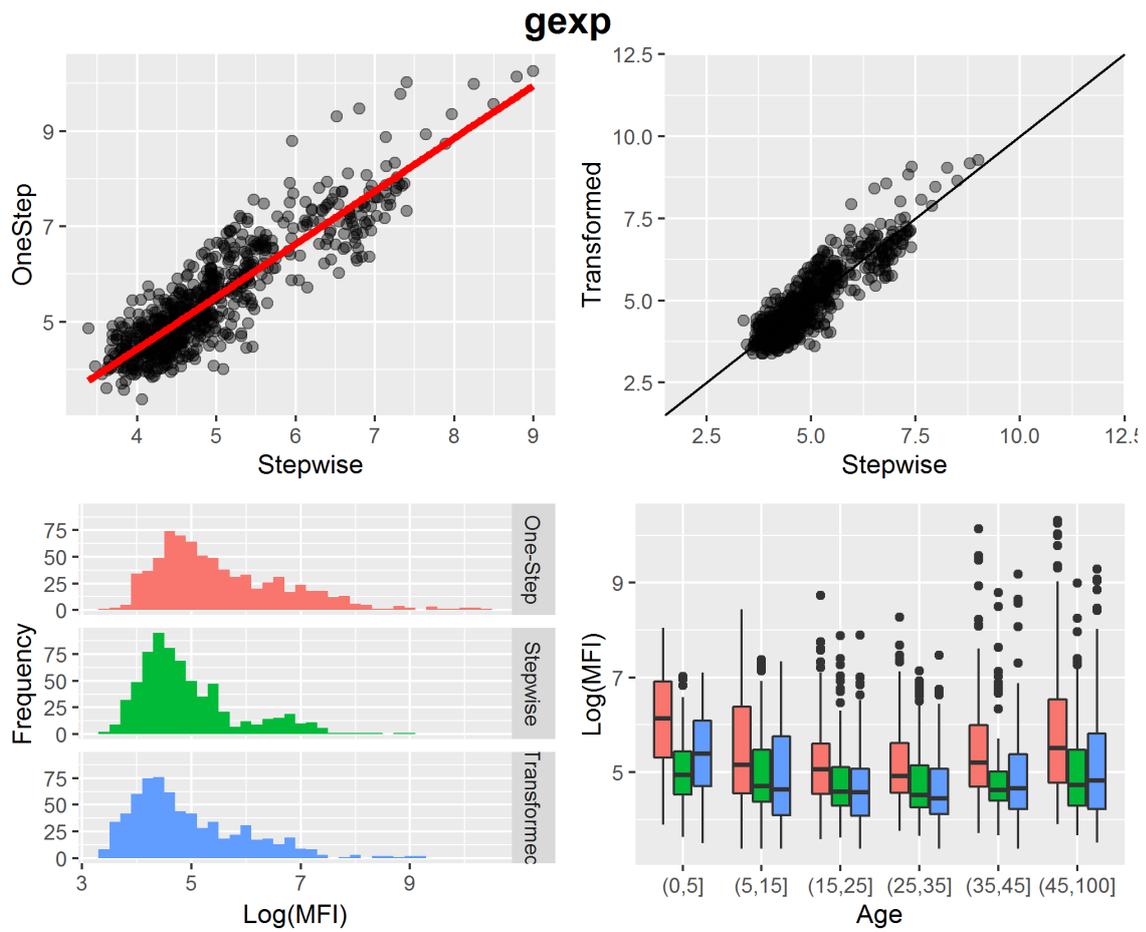
Transformation of antibody responses between the OneStep and Stepwise multiplex bead assay protocols for GEX-P. For legend see page 218.
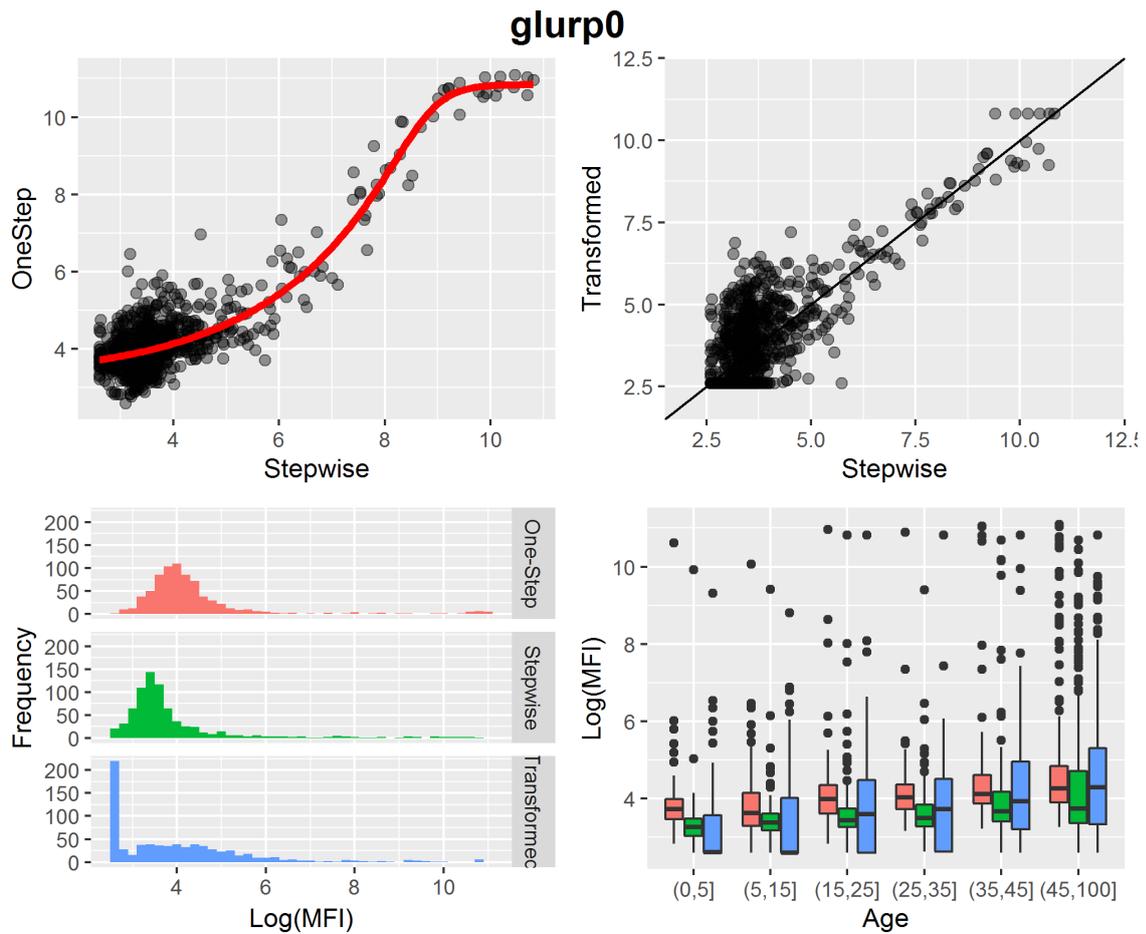
Transformation of antibody responses between the OneStep and Stepwise multiplex bead assay protocols for GLURP-R0. For legend see page 218.
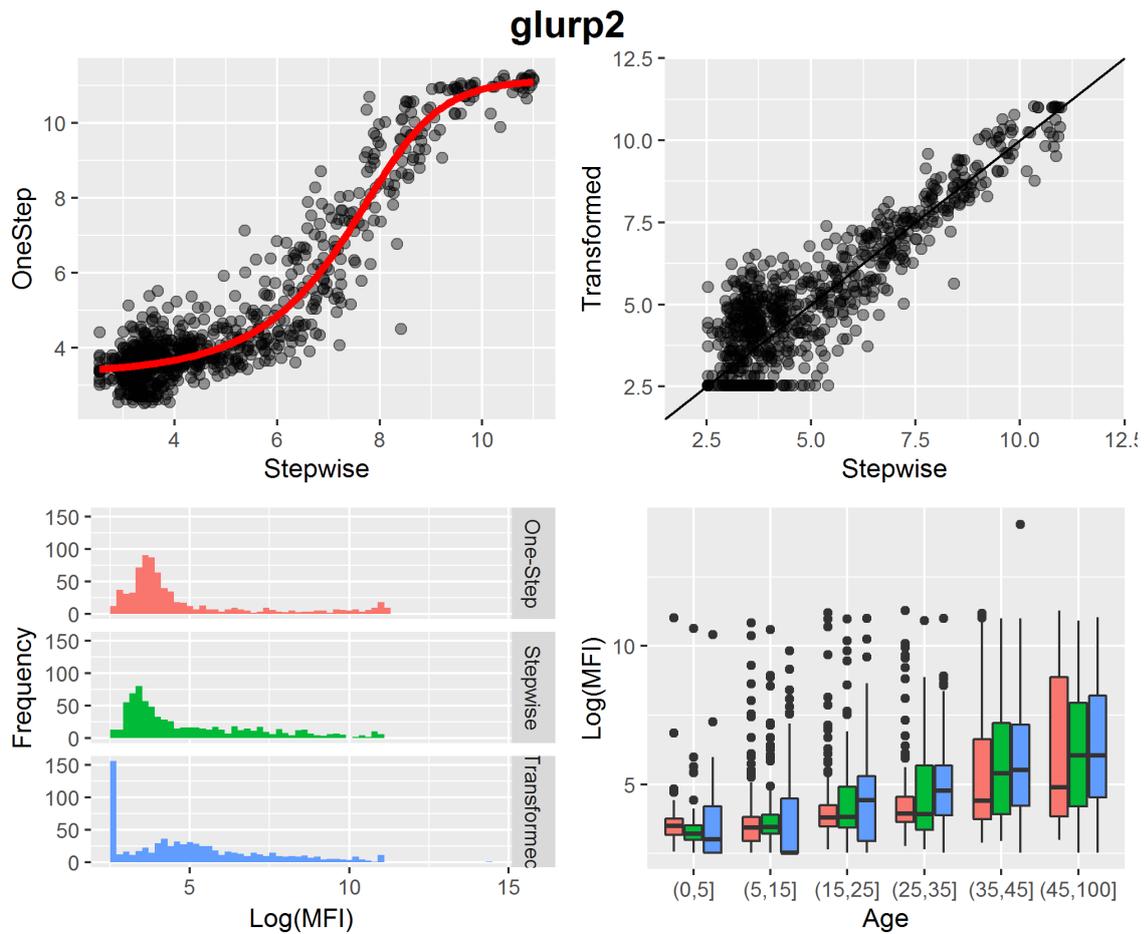
glurp2

**Transformation of antibody responses between the OneStep and Stepwise multiplex bead assay protocols for GLURP-R2.** For legend see page 218.
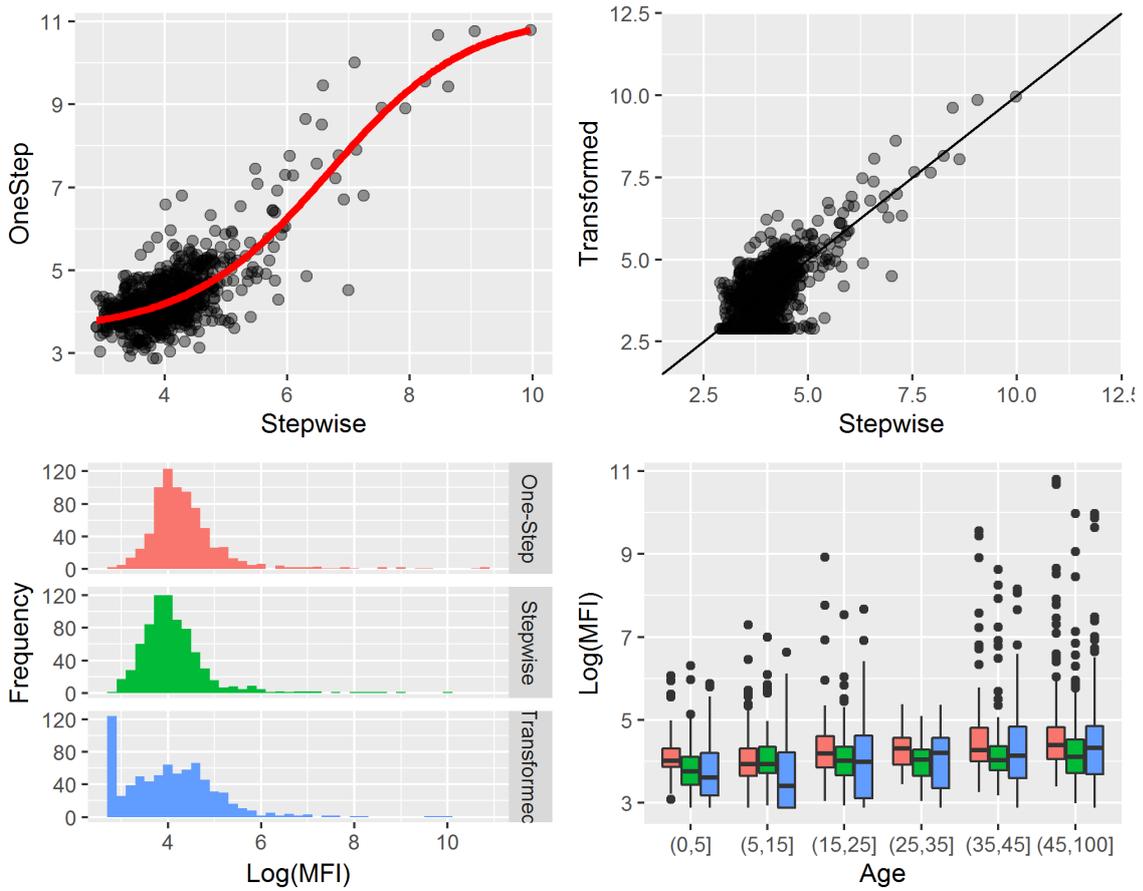
Transformation of antibody responses between the OneStep and Stepwise multiplex bead assay protocols for H103. For legend see page 218.
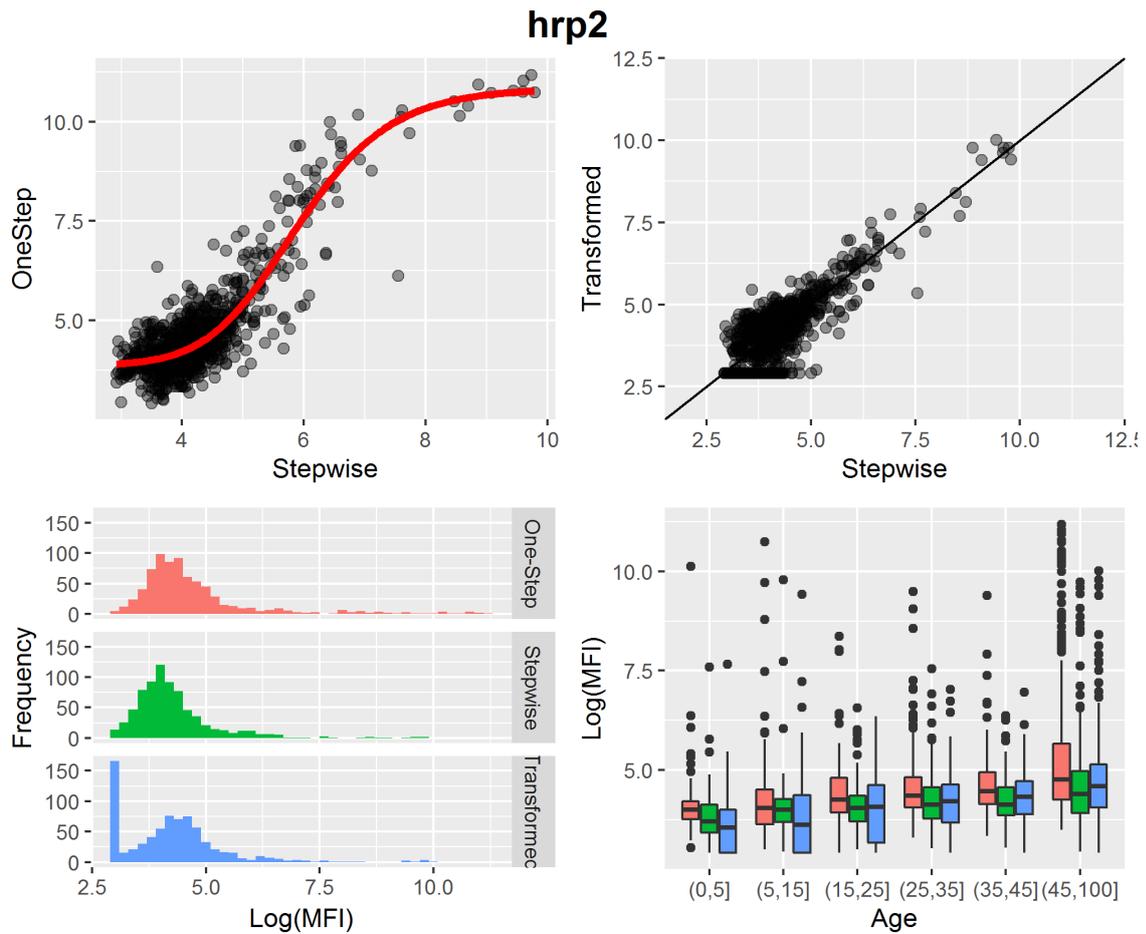
hrp2

Transformation of antibody responses between the OneStep and Stepwise multiplex bead assay protocols for HRP2. For legend see page 218.
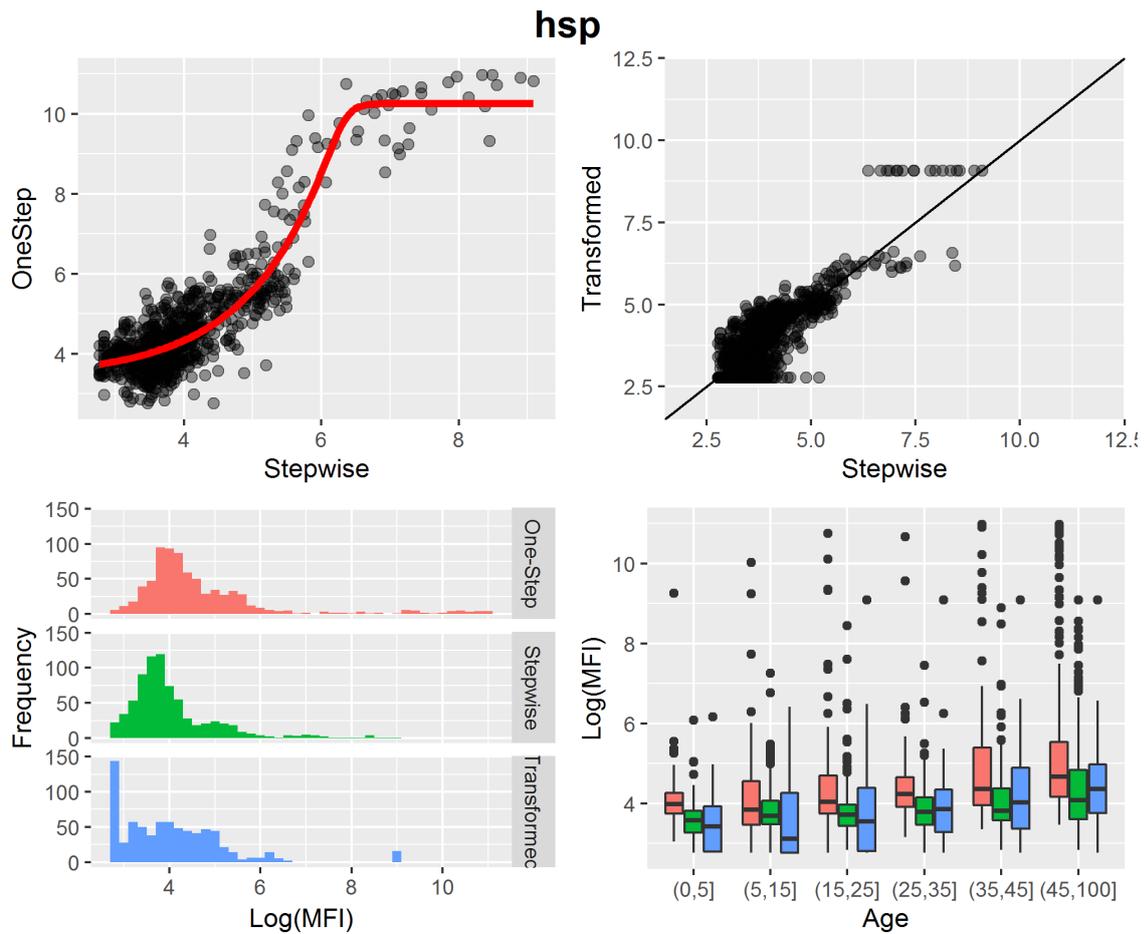
Transformation of antibody responses between the OneStep and Stepwise multiplex bead assay protocols for HSP40. For legend see page 218.
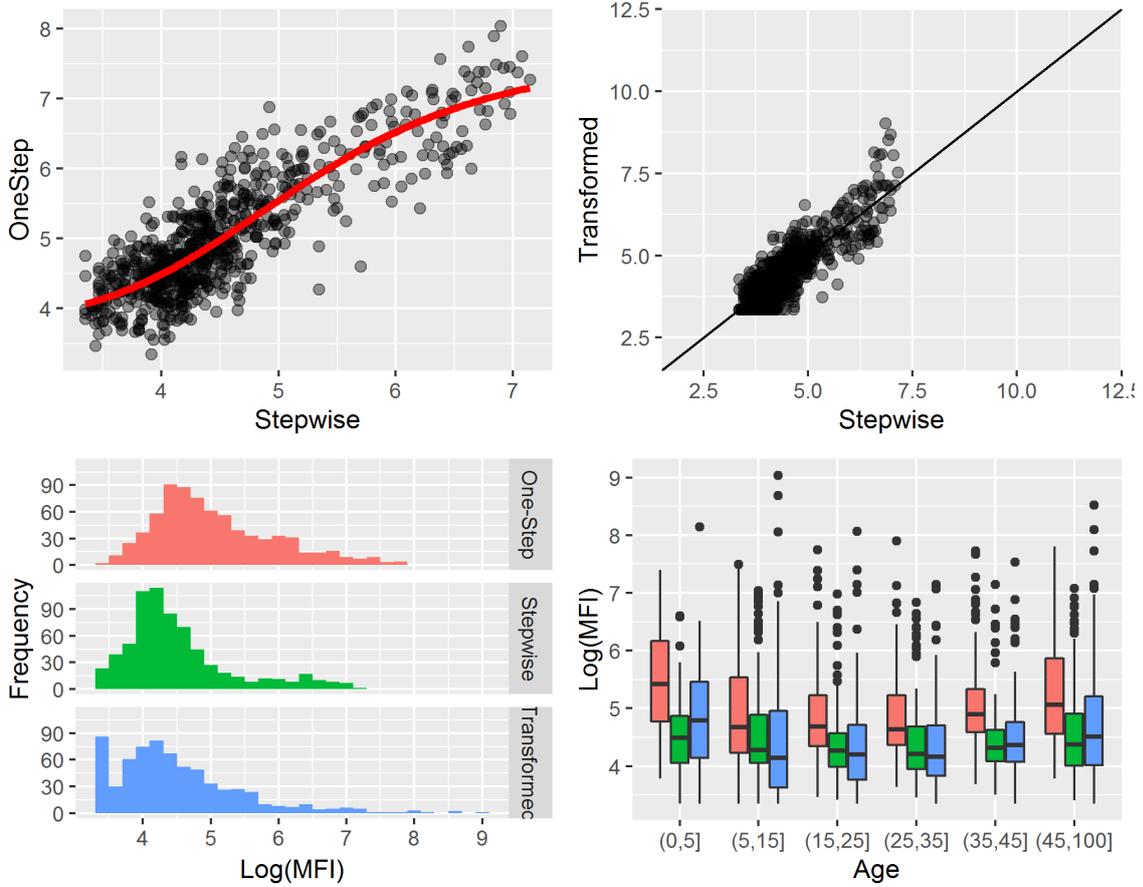
Transformation of antibody responses between the OneStep and Stepwise multiplex bead assay protocols for Hyp2. For legend see page 218.
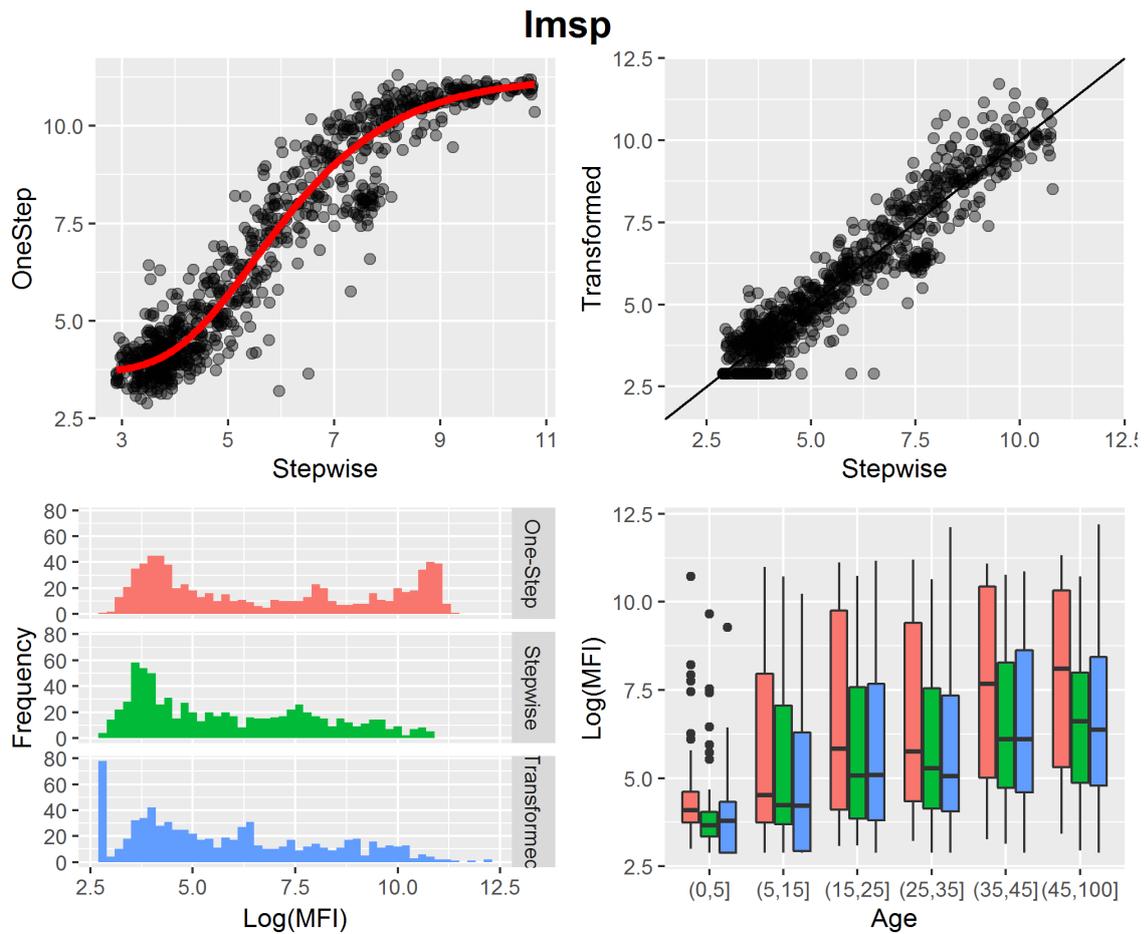
Imsp

Transformation of antibody responses between the OneStep and Stepwise multiplex bead assay protocols for MSP1-19. For legend see page 218.
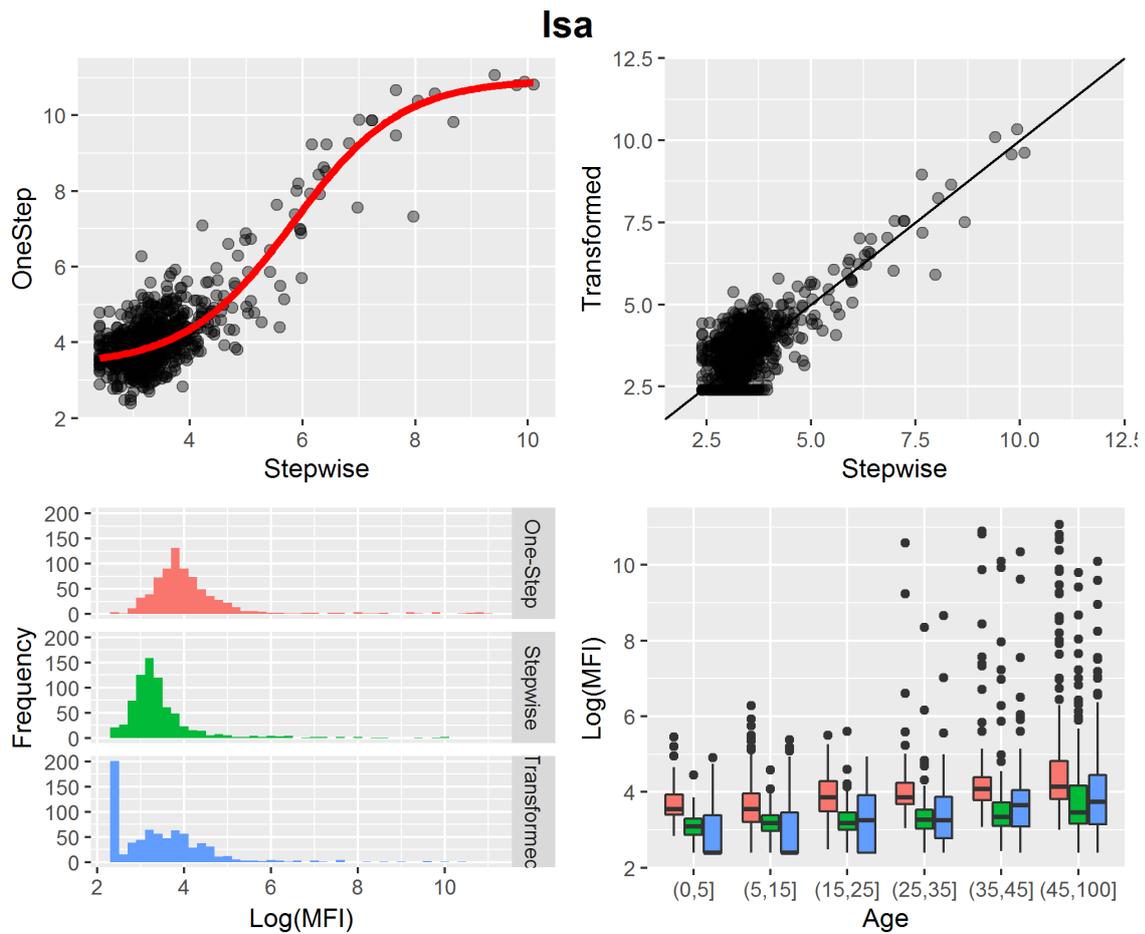
Transformation of antibody responses between the OneStep and Stepwise multiplex bead assay protocols for LSA-1. For legend see page 218.
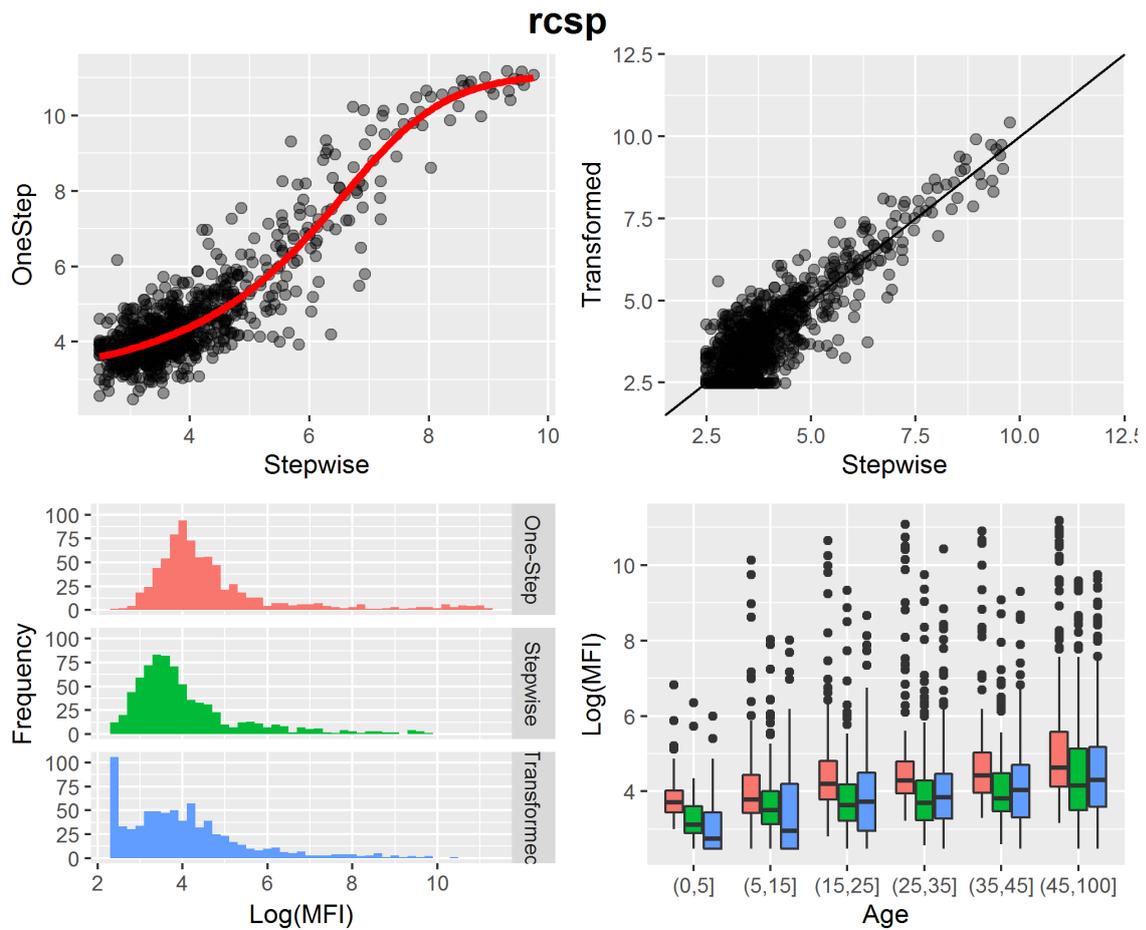
Transformation of antibody responses between the OneStep and Stepwise multiplex bead assay protocols for rCSP. For legend see page 218.
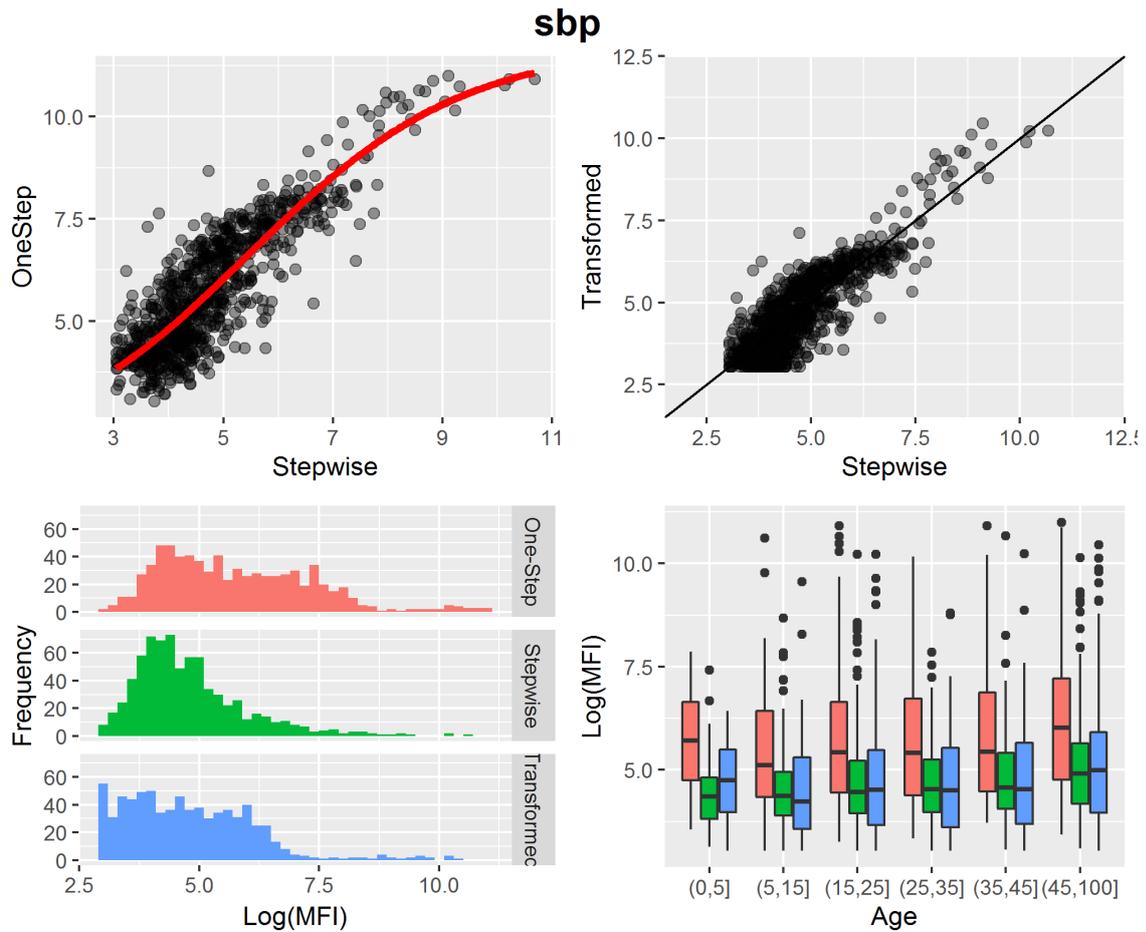
**Transformation of antibody responses between the OneStep and Stepwise multiplex bead assay protocols for SBP1.** For legend see page 218.
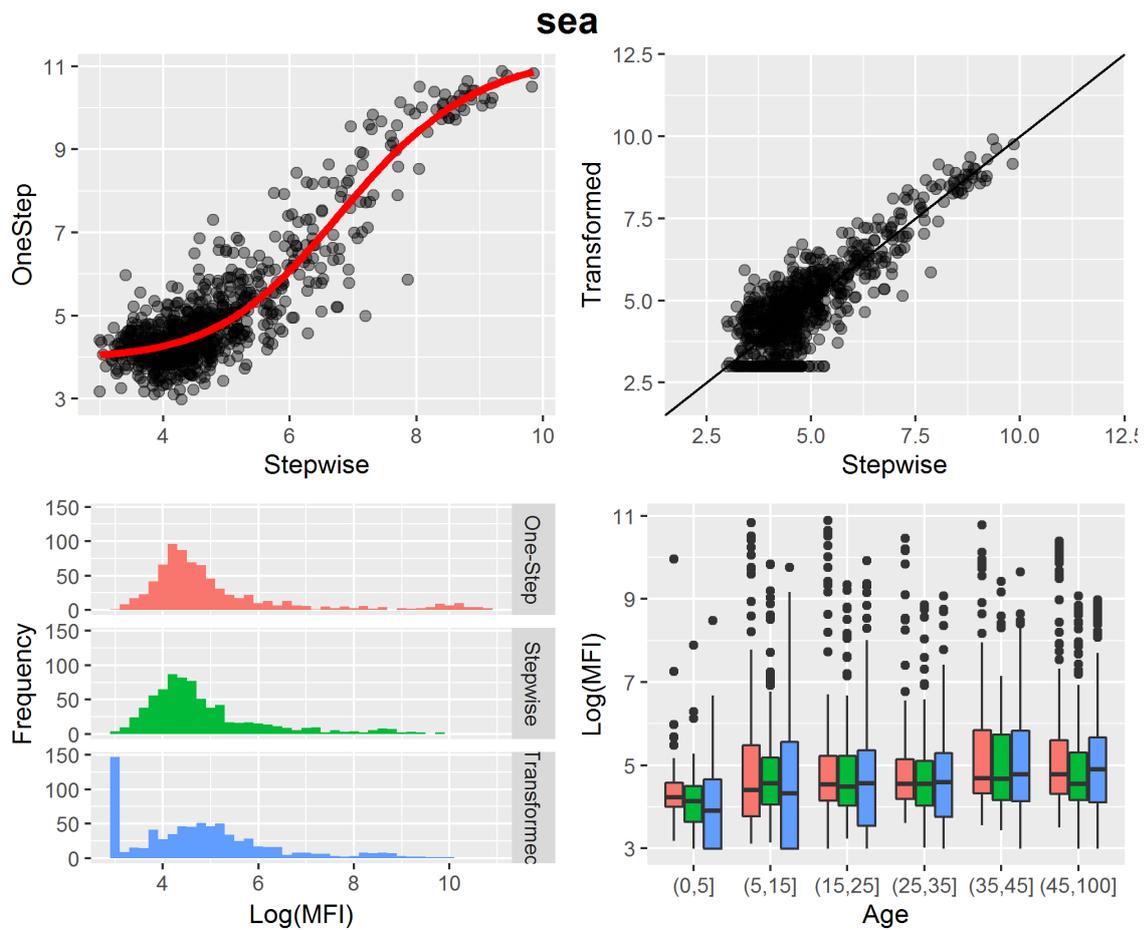
**Transformation of antibody responses between the OneStep and Stepwise multiplex bead assay protocols for SEA.** For legend see page 218.

Appendix C: Instruction Manuals of Commercially Available Enzyme-linked Immunosorbent Assays

Trinity Biotech

CAPTIA™ Malaria EIA

| REF | 2322470 | Captia™ Malaria EIA 96 Test Kit |
| REF | 2322471 | Captia™ Malaria EIA 480 Test Kit |

Pour d'autres langues	Para outras línguas	
Für andere Sprachen	Για τις άλλες λιώσσες	
Para otras lenguas	För andra språk	
Per le altre lingue	For andre språk	
Dla innych języków	For andre sprog	**www.trinitybiotech.com**

INTENDED USE

This kit is intended for use by appropriately trained and qualified personnel for the qualitative and semi-quantitative detection of antibodies to *Plasmodium falciparum (P. falciparum), Plasmodium vivax (P. vivax), Plasmodium ovale (P. ovale)* and *Plasmodium malariae (P. malariae)* in human serum and plasma.

SUMMARY AND PRINCIPLE

Malaria is one of the most common diseases in the world. More than half the world population lives in malaria-infected areas. Over 200 million cases annually result in up to 3 million deaths each year; a majority of which are in young children. In non-endemic areas, it is one of the most important imported diseases, resulting in a number of deaths in late-diagnosed or unsuspected cases each year.

The disease is caused by protozoa of the genus *Plasmodium*, transmitted by the bite of the female *Anopheles* mosquito. There are four species causing human malaria: *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale* and *Plasmodium malariae*. The disease may also be transmitted by transfusion of infected blood. Once in the blood the sporozoite makes its way to the liver where for the next two weeks merozoites are produced. These are released into the blood where they invade the red cells and produce more merozoites, causing the cells to rupture. It is this rupturing that is responsible for the clinical symptoms.

Of the four species, *P. falciparum* is the most common and the most virulent, causing most malaria-related deaths. *P. vivax* is the next most common cause of malaria. Although rarely fatal, this form of malaria can be accompanied by severe clinical symptoms. It is a common cause of malaria in South East Asia and South America.

People infected with *Plasmodium* spp. form antibodies in response. Captia™ Malaria is designed to detect antibodies occurring in subjects infected with *P. falciparum, P. vivax, P. ovale* and *P. malariae*.

Captia™ Malaria EIA uses four recombinant antigens in a sandwich test to produce a test that is both highly specific and sensitive. The antigens will detect *P falciparum, P. vivax, P. ovale* and *P. malariae*-specific IgG, IgM, and IgA, enabling the test to detect antibodies during all stages of infection. All reagents except the Conjugate and Wash solution are supplied ready to use and are colour coded. The procedure uses undiluted samples and standard volumes for ease of both manual and automated use. The assay can be used with both serum and plasma.

The plastic wells are coated with a mixture of *P. falciparum* and *P. vivax* recombinant antigens. The antigenic similarity between *Plasmodium* species means that antibodies to all species can be detected. Specific antibodies in serum or plasma specimens combine with these antigens and with the same antigens conjugated to horseradish peroxidase, when conjugate is added to a well in which the specimen has been incubated. After unreacted material has been removed by washing, the presence of bound enzyme indicating the presence in the specimen of specific antibodies is revealed by a colour change in the substrate/chromogen mixture. The intensity of the colour is compared to that in control wells to determine the presence or absence of specific antibody.

REAGENTS

REAGENT DESCRIPTION

R1: Plate	Polystyrene coated with recombinant antigens. 96 wells in 12 strips of 8. 1 plate per 96 test kit and 5 plates per 480 test kit.
R2: Positive control	Positive human serum. 1.5 mL. Red.
R3: Negative control	Negative human serum. 2 mL. Yellow.
R4: Conjugate	11x concentrate. Recombinant antigens conjugated to horseradish peroxidase. Blue/Colourless. 0.8 mL per 96 test kit and 3 mL per 480 test kit.
R5: Conjugate Buffer	Buffered saline containing surfactant and stabilisers. Green. 8 mL per buffer 96 test kit and 30 mL per 480 test kit.
R6: Substrate	Urea peroxide and tetramethyl benzidine. Pink. 7 mL per 96 Test kit and 30 mL per 480 test kit.
R7: Wash	20x concentrated. Saline containing surfactant. Colourless. 125 mL per 96 test kit and 2x 125 mL per 480 test kit.
R8: Stop solution	0.5 M H_2SO_4. Colourless. 7 mL per 96 test kit and 30 mL per 480 test kit.

Instructions for Use.
Bag for storing unused wells.

ADDITIONAL MATERIALS REQUIRED

- Properly calibrated and maintained pipetting devices capable of delivering volumes of 50 µL (specimens and reagents) and approximately 300 µL (wash fluids).
- Plate or strip reader to read at 450 nm and (optionally) at a wavelength between 620 and 690 nm.
- 37°C incubator.
- Plate shaker

INSTRUMENTS

Captia™ Malaria may be automated for both liquid handling and result interpretation. A variety of systems have been used for this – please consult the manufacturers of both the kit and the automation system for advice on automation.
Equipment should be able to support the following tolerances:

Volume dispensed	+/- 10%
Incubation temperature	+/- 2°C
Incubation time	+/- 2 minutes

STORAGE AND STABILITY

- All reagents as supplied may be used up to their expiry date if stored at 2-8°C.
- Store bottles upright.
- Do not freeze.
- Do not expose substrate to direct sunlight.
- Diluted Wash is stable for 4 weeks at 2-8°C.
- Unused coated strips are stable for 4 weeks at 2-8°C if stored in the re-sealable bag provided.
- Diluted conjugate is stable for 4 weeks at 2-8°C.

SPECIMEN COLLECTION AND STORAGE

- Serum or plasma (collected into EDTA, sodium citrate or heparin) samples may be used.
- Specimens may be stored at 2-8°C for up to 7 days before testing.
- Specimens needing longer storage should be frozen at -20°C or lower and be well mixed after thawing.
- Samples that are contaminated with bacteria or contain visible particulate matter should NOT be tested.

WARNINGS AND PRECAUTIONS

- **For *in-vitro* diagnostic use only.**
- For professional use only.
- This test is designed to be used by appropriately trained laboratory personnel in the clinical laboratory.
- All human materials used have been tested and found negative for indicators of HIV, Hepatitis B, and Hepatitis C infection. However, they should be treated as potentially infectious and treated and discarded according to applicable regulations for such materials.
- Blood samples may contain pathogenic organisms. Gloves should be worn throughout, and all discarded materials sterilised by appropriate methods.
- All equipment should be properly maintained and calibrated according to the manufacturers' instructions.
- Do not combine or interchange reagents from kits with different lot numbers.
- Ensure that bottle caps are returned to the correct bottles.
- Do not use the kit after its expiry date

PROCEDURE

PROCEDURAL NOTES AND PRECAUTIONS

- Bring all reagents and specimens to room temperature prior to use.
- Washing must be thorough, with complete filling and emptying of the wells at each cycle.
- The Negative control must be tested three times with each lot of tests, and the Positive control twice.

REAGENT PREPARATION

- Dilute Wash (R7) 1 in 20 with distilled or deionised water prior to use.
- Dilute conjugate (R4) 1 + 10 in Conjugate Buffer (R5) (50 µL + 500 µL per 10 wells).

TEST PROCEDURE

1. Add **50 µL of the undiluted sample** (or control (R2 or R3) – see "Procedural Notes and Precautions" above) to a coated well.
2. Mix on a plate shaker for 30 seconds.
3. Incubate (covered) at 37°C for 30 minutes.
4. Wash 5x with working strength wash. A short soak time of about 30 seconds is recommended between each wash cycle. Tap out excess liquid.
5. Add **50 µL diluted conjugate (R4 + R5)** to each well.
6. Incubate (covered) at 37°C for 30 minutes.
7. Wash 5x with working strength wash. A short soak time of about 30 seconds is recommended between each wash cycle. Tap out excess liquid.
8. Add **50 µL substrate/chromogen (R6)** mixture to each well.
9. Incubate at room temperature for 30 minutes. As the substrate is photosensitive, it is recommended that the plate be protected from light during this incubation.
10. Add **50 µL Stop solution (R8)** to each well (blue colour changes to yellow).
11. Read results at 450 nm (A_{450}). Use of a reference filter at 620–690 nm will eliminate effects of scratches, bubbles, etc.

Verification of Sample and Reagent addition
Automatic Reading:
Addition of samples is verified by reading at 450 nm, using a reference filter at 620nm. A well with sample added will have an A_{450} reading of ≥0.050.
Addition of conjugate is verified by reading at 450 nm. A well with conjugate added must have an A_{450} reading of >0.2.
Addition of substrate is verified by reading at 550 nm. A well with substrate added must have an A_{550} reading of >0.080.

RESULTS

ASSAY VALIDATION

A_{450} of each Negative Control should be lower than or equal to 0.080. If one control is above this value the reading should be ignored and the cut–off calculated using the remaining two.
A_{450} of each Positive Control should be greater than or equal to 1.000.

CUT-OFF CALCULATION

The Cut-Off Point (COP) is calculated as the mean of the Negative controls (NC) + 0.100 absorbance units.

i.e. $\dfrac{(NC1 + NC2 + NC3)}{3} + 0.100$

Example: $\dfrac{0.030 + 0.025 + 0.035}{3} = 0.030$

∴ Cut-Off Point = 0.030 + 0.100 = 0.130

INTERPRETATION OF RESULTS

Samples with an A_{450} value greater than the cut-off point are considered positive by Captia™ Malaria.

Samples with an A_{450} value less than the cut-off point are considered negative by Captia™ Malaria.

Samples just below the cut-off (C.O. –10% A_{450}) should however, be interpreted with caution. It is advisable to retest the corresponding samples in duplicate when the systems and laboratory procedures permit.

Re-tested samples that are above the cut-off in at least one duplicate are considered positive and should be investigated further. Samples that are below the cut-off in both duplicates are considered to be negative.

PERFORMANCE CHARACTERISTICS

Specificity
External data from 13,608 donor samples deemed at risk to malaria infection gave 96.1% specificity (95% confidence limits 95.8-96.4%).

Sensitivity
External data for 76 acute *P. falciparum* cases showed 92.5% sensitivity (95% confidence limits 83.6-97.1%)

External data for 258 IFAT ≥80 for *P. falciparum* showed 94.2% sensitivity (95% confidence limits 90.6-96.7%)

Internal data for *P. vivax* showed 100% sensitivity (95% confidence limits 59-100%)

Only small numbers of samples from *P. ovale* and *P. malariae* infections have been studied. Sensitivity for these was 80% and 67% respectively. Numbers were too small to allow meaningful statistical analysis.

Precision

Specimen No.	No. of replicates	Mean A450-A620	Intra-assay CV (%)	Inter-assay CV (%)
1	16	2.402	2.28	3.78
2	16	1.316	3.83	5.17
3	16	0.672	3.83	5.52
4	16	0.353	4.06	6.15
5	16	0.195	3.19	6.16
6 (Negative)	16	0.046	6.95	6.84

REFERENCES

1. Kitchen A.D. et al Evaluation of a malarial antibody assay for use in the screening of blood and tissue products for clinical use Vox Sanguinis (2004) **87**, 150-155
2. Seed C.R. et al The efficacy of a malarial antibody enzyme immunoassay for establishing the reinstatement status of blood donors potentially exposed to malaria Vox Sanguinis (2005) **88**, 98-106
3. Kitchen A.D. et al Tranfusion transmitted malaria: current donor selection guidelines are not sufficient Vox Sanguinis (2005) **88**, 200–201

ORDERING INFORMATION

KIT

Kit Content	Item	Quantity
2322470	Captia™ Malaria	96 Tests
2322471	Captia™ Malaria	480 Tests

GUIDE TO SYMBOLS

Consult Instructions for Use

Temperature limitation

REF — Catalog number

IVD — For *in vitro* Diagnostic Use

Manufacturer

LOT — Batch code

Use by

CE

PLATE — Coated microtitre plate

WASH x20 — Concentrated wash buffer

CONTROL - — Negative control

CONTROL + — Positive control

CONJUGATE x11 — Conjugate

CONJUGATE BUFFER — Conjugate buffer

SUBSTRATE — Substrate

STOP — Stop solution

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EC REP

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2470-29 Rev D
01/2015

236

newbio – Malaria TA

An EIA assay for the qualitative and semi-quantitative detection of antibodies to *P.falciparum, P.vivax, P.Malariae and P. Ovale*

CE

INTRODUCTION AND INTENDED USE

Malaria is one of the most common diseases in the world. More than half the world population lives in malaria-infected areas. Over 200 million cases annually result in up to 3 million deaths each year; a majority of which are in young children. In non-endemic areas, it is one of the most important imported diseases, resulting in a number of deaths in late-diagnosed or unsuspected cases each year.

The disease is caused by protozoa of the genus Plasmodium, transmitted by the bite of the female Anopheles mosquito. There are four species causing human malaria: P.falciparum, P.vivax, P. malariae, and P. ovale. The disease may also be transmitted by transfusion of infected blood. Once in the blood the sporozoite makes its way to the liver where for the next 2 weeks merozoites are produced. These are released into the blood where they invade the red cells and produce more merozoites, causing the cells to rupture. It is this rupturing that is responsible for the clinical symptoms.

Of the four species, P. falciparum is the most common and the most virulent, causing most malaria-related deaths. P. vivax is the next most common cause of malaria. Although rarely fatal, this form of malaria can be accompanied by severe clinical symptoms. It is a common cause of malaria in S.E. Asia and S. America.

People infected with Plasmodium spp. form antibodies in response. The Newmarket Biomedical MALARIA EIA kit is designed to detect antibodies occurring in subjects infected with P. falciparum and P. vivax, P. malariae, and P. ovale.

PRINCIPLE OF THE TEST

newbio-Malaria TA is a two step sandwich assay using specific recombinant antigens for all four species ensuring enhanced sensitivity and specificity. The assay is for use with plasma and serum samples and will detect antibodies at all stages of infection. Antibodies are captured by recombinant antigens on the plate and marked by HRP recombinant conjugates for visualisation with TMB substrate. The reagents and protocol ensure ease of use and assay control.

CONTENTS

Name	Description	96 t NB016	480 t NB017
Positive Control	Human antiserum diluted in stabilisation buffer	1 mL	4 mL
Negative control	Rabbit serum diluted in stabilisation buffer	1 mL	4 mL
Plate	12 x 8 well strips coated recombinant antigen	x 1	x 5
Conjugate	HRP conjugated recombinant antigen	8 mL	30 mL
Substrate	TMB/Peroxidase in stabilisation buffer	7 mL	30 mL
Stop solution	0.5M Sulphuric acid	8 mL	30 mL
Wash buffer	20 x Concentrated	125 mL	250 mL

WARNINGS AND PRECAUTIONS

For *in-vitro* diagnostic use only.
Material of human origin has been tested negative by FDA Approved methods for HIV 1&2, HCV antibodies and HBsAg.
All human samples should be handled as if capable of transmitting disease and disposed of according to local guidelines.

STORAGE
Store at **2–8°C**.
Substrate is light sensitive.

LIMITATIONS OF USE
newbio-Malaria TA may be used for neat serum and plasma.
Do not use after the stated expiry date.
Do not use substrate which has turned blue.
Controls containing sodium azide are not valid.

SAMPLES

Use fresh serum or plasma samples free of microbial contamination. Samples may be stored at 2-8°C for up to 7 days prior to testing. Samples can be frozen at -20°C or lower - these should be thawed and mixed prior to testing.

ASSAY PROCEDURE

Equipment Required

Micro-pipettes capable of delivering: 50 and 300µL
Plate reader
Incubator
newbio-Malaria TA may be used in combination with automated assay equipment. Consult manufacturers for advice.

Bring all reagents and samples to room temperature before use.

Dilute wash buffer in deionised water 1/20 prior to use.

Kit controls must be run with each assay.

The kit positive should be run in duplicate.
The kit negative should be run in triplicate.

Substrate verification

The addition of the coloured substrate can be verified visually or by reading the wells at 550nm.
The OD will be ≥ 0.08

Protocol

1. Add 50µl neat sample or Kit Control to reaction well.

   Cover the plate and incubate at **37°C for 30 minutes**.

2. Wash strips x 5 with diluted wash buffer.

   Use a minimum of 300µl per wash.

   Ensure excess wash is removed.

3. Add 50µl of HRP Conjugate to each reaction well, mix for 20 seconds.

   Cover the plate and incubate at **37°C for 30 minutes.**

4. Wash strips x 5 with diluted wash buffer.

   Use a minimum of 300µl per wash.

   Ensure excess wash is removed

5. Add 50µl of TMB substrate to each reaction well.

> Cover the plate from light and incubate at **RT for 30 minutes**.

6. Add 50µl of Stop to reaction wells.

7. Read wells at 450nm (reference filter 620–690nm)

> Read within 30 minutes of addition of stop.

Assay Validation

Kit Negative:

The assay is valid if the $A_{450-620}$ value of each control reading is **$\leq$ 0.080**

If one of the values is above 0.080 then the remaining values should be used in the cut off calculation.

Kit Positive:

The value of each control reading should be **$\geq$ 0.500**

Cut-off Value

The cut off is 0.100 plus the mean of the negative values:
> 0.100 + mean (N1 + N2 + N3)

Example: 0.100 + mean (0.020 + 0.022 + 0.021) = 0.121

Interpretation

Negative

Samples with an OD less than the calculated cut off value are considered negative**.**

Positive

Samples with an OD greater than or equal to the calculated cut off value are considered positive and should be retested in duplicate.

Where both retests are below the cut off then the sample should be considered negative.

Where one of the retests is equal to or above the calculated cut off then it should be considered as a positive result and submitted for further investigation.

PERFORMANCE CHARACTERISTICS

Clinical Specificity

A study on 450 donor plasma showed 100% specificity. (95% confidence limits 98.8 – 100 %)

Clinical Sensitivity

Species	Sample No	Reactive	% Sensitivity
Falciparum	410	400	97.6
Malariae	14	14	100.0
Ovale	40	40	100.0
Vivax	64	62	96.9
All species	**528**	**516**	**97.7**

(All Species 95% confidence limits 96.1 – 98.7%)

Analytical Sensitivity

newbio-Malaria TA has a sensitivity of 1 IU/ml against the 1st IS for Anti-malaria (Plasmodium falciparum) human serum NIBSC code: 10/198

Analytical Specificity

No cross reaction was seen with samples of the following groups:

Rhumatoid factor,
Lyme disease,
Toxoplasma,
EBV ,
SLE (Lupus).
Pregnant women
Multipara women
Hepatitis A
Hepatitis B
Hepatitis C
HTLV I/II
HIV ½

Interferences

Interferences with hemolytic, lipemic and icteric sera or sera with high albumin are not observed up to a concentration of 2 mg/ml hemoglobin, 33 mg/ml triglycerides 0.2 mg/ml bilirubin and 60mg/ml human albumin.

KEY TO SYMBOLS

REF	Catalogue number
IVD	IVD In Vitro Diagnostic Medical Device
	Manufactured by
	Temperature limitation
	Use by
LOT	Batch code
	Consult instructions for use

238

Malaria Ab

3rd Generation Enzyme Immunoassay for the determination of antibodies to Plasmodium species in human serum and plasma

- for "in vitro" diagnostic use only -
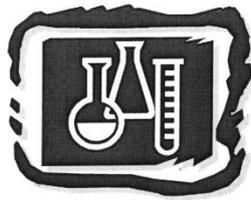
DIA.PRO
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Fax +39 02 26007726
e-mail: info@diapro.it

Code MALAB.CE
96/192/480/960 Tests

Malaria Ab

A. INTENDED USE

Enzyme Immunoassay (ELISA) for the determination of antibodies to Plasmodium species in human sera and plasma.

The kit is intended for the screening of blood units and the identification of people that came into contact with the protozoa and developed an immunological response.

The kit is for in vitro diagnostic use only and the test has to be carried out by professional people, opportunely trained.

B. INTRODUCTION

Plasmodium species are obligate intracellular protozoa related to Babesia and Toxoplasma. Plasmodium species reproduce sexually in mosquitoes; mosquitoes transmit the resulting sporozoites into humans where the organisms reproduce asexually. The sporozoites multiply within the liver; resulting merozoites invade erythrocytes where the merozoites multiply or mature into male and female gametocytes which eventually will be taken up by a mosquito during a blood meal.

P.falciparum and P.vivax cause approximately 80% and 15%, respectively, of all cases of Malaria.

Malaria is the most severe infectious disease of tropical and subtropical areas of the world that still is heavily affecting millions of people and generating millions of casualties.

The detection of anti P.species antibodies can identify in suspected individuals a case of recent or past malaria.

C. PRINCIPLE OF THE TEST

Recombinant proteins representing immunodominant epitopes of Plasmodium species, are coated onto wells of a microplate.

Recombinant proteins have been carefully selected to ensure the screening of all antibodies to P.species. Serum or plasma samples are added to these wells and, if antibodies specific to P.species (IgG, IgM or IgA) are present in the sample, they will form stable complexes with the recombinant antigens in the well.

Antigen-antibody complexes are then identified through the successive addition of: (1) same biotinylated recombinant proteins specific to P.species and; (2) horseradish peroxidase HRP Streptavidin conjugate.

The hydrolytic activity of horseradish peroxidase allows for the quantification of these antibody-antigen complexes.

Peroxidase substrate solution is then added.

During incubation, a blue colour will develop in proportion to the amount of anti P.species antibodies bound to the well, thus establishing their presence or absence in the sample. Wells containing samples negative for anti-P.species antibody remain colourless.

A stop solution is added to each well and the resulting yellow colour is read on a microplate reader at 450 nm.

D. COMPONENTS

Code MALAB.CE contains reagents for 96/192/480/960 tests.
The following describes the composition of the standard format (192 tests/kit).

1. Microplate MICROPLATE

n° 2 microplates. 12 strips of 8 breakable wells coated with Plasmodium species specific recombinant antigens. Plates are sealed into a bag with desiccant.

2. Negative Control CONTROL -

1x4.0ml/vial. Ready to use control. It contains human serum negative for P.species antibodies and 0.1% Kathon GC as preservatives. The negative control is pale yellow color coded.

3. Positive Control CONTROL +

1x4.0ml/vial. Ready to use control. It contains human serum positive for P.species antibodies and 0.1% Kathon GC as preservatives. The Positive Control is light green color coded.

Important Note: Even if this component has been treated with chemicals able to inactivate P.species, this does not fully ensure the absence of viable pathogens, and therefore, the control should be handled as potentially bio hazardous, in accordance with good laboratory practices.

4. Calibrator: CAL ....

2 vials. Dissolve carefully the content of the lyophilized vial with the volume of EIA grade water reported on its label. Mix well on vortex before use.

Important Notes:
1) When dissolved the Calibrator is not stable. Store in aliquots at –20°C.
2) Even if this component has been treated with chemicals able to inactivate P.species, this does not fully ensure the absence of viable pathogens, and therefore, the control should be handled as potentially bio hazardous, in accordance with good laboratory practices.

5. Wash buffer concentrate WASHBUF 20X

2x60ml/bottle. 20x concentrated solution. It contains 0.1% Kathon GC. Once diluted, the wash solution contains 10 mM phosphate buffer saline pH 7.0+/-0.2 and 0.05% Tween 20.

6. Conjugate # 1 CONJ 1

8 vials. The vial contains lyophilized biotinylated P.species recombinant antigens. Vials are to be fully dissolved with 6 ml of the conjugate 1 diluent.

7. Conjugate 1 Diluent CONJ 1 DIL

1x60ml/bottle. Used to dissolve the lyophilized powder of Conjugate # 1, it contains Tris saline Buffer supplemented with 0.05% Kathon GC, Tween 20 and BSA.

8. Conjugate # 2 CONJ 2

1x30ml/bottle. The solution contains HRP conjugated with streptavidin in Tris saline Buffer supplemented with 0.05% Kathon GC, Tween 20 and BSA. This component is colour coded in red.

9. Chromogen/Substrate SUBS TMB

1x50ml/bottle. Ready-to-use component. It contains 50 mM citrate buffer pH 3.5-3.8, 4% dimethylsulphoxide, 0.03% tetra-methyl-benzidine or TMB and 0.02% hydrogen peroxide or H_2O_2.

Note: To be stored protected from light as sensitive to strong illumination.

10. Sulphuric Acid H_2SO_4 O.3 M

1x32ml/vial. It contains 0.3 M H_2SO_4 solution.
Attention: Irritant (Xi R36/38; S2/26/30).

11. Sample Diluent DILSPE

1x14ml/vial. Contains Tris buffer supplemented with 0.05% Kathon GC and Tween 20; used for specimen dilution. This component is colour coded in light blue.

12. Plate sealing foils   n° 4

13. Package insert   n° 1

Important note: Only upon specific request, Dia.Pro can supply reagents for 96, 480, 960 tests , as reported below :

1.Microplate	n°1	n°5	n°10
2.Negative Control	1x2.0ml/vial	1x10ml/vial	1x20ml/vial
3.Positive Control	1x2.0ml/vial	1x10ml/vial	1x20ml/vial
4.Calibrator	n°1 vial	n°5 vials	n°10 vials
5.Wash buff conc	1x60ml/bottle	5x60ml/bottles	4x150ml/bottles
6.Conjugate # 1	n° 4 vials	n°20 vials	n°40 vials
7.Conjugate 1 Diluent	1x30ml/vial	3x50ml/bottle	2x150ml/bottles
8.Conjugate # 2	1x15ml/vial	2x38ml/bottle	2x75ml/bottle
9.Chrom/Substrate	1x25ml/vial	3x42ml/bottle	2x125ml/bottles
10.Sulphuric Acid	1x15ml/vial	2x40ml/bottles	2x120ml/bottles
11.SampleDiluent	1x7ml/vial	1x35ml/bottle	1x70ml/bottle
Plate seal foils	n° 2	n° 10	n° 20
Pack. insert	n° 1	n°1	n° 1
Number of tests	**96**	**480**	**960**
Code	**MALAB.CE.96**	**MALAB.CE.480**	**MALAB.CE.960**

E. MATERIALS REQUIRED BUT NOT PROVIDED

1. Calibrated Micropipettes (200ul and 10ul) and disposable plastic tips.
2. EIA grade water (bidistilled or deionised, charcoal treated to remove oxidizing chemicals used as disinfectants).
3. Timer with 60 minute range or higher.
4. Absorbent paper tissues.
5. Calibrated ELISA microplate thermostatic incubator capable to provide a temperature of +37°C.
6. Calibrated ELISA microwell reader with 450nm (reading) and with 620-630nm (blanking) filters.
7. Calibrated ELISA microplate washer.
8. Vortex or similar mixing tools.

F. WARNINGS AND PRECAUTIONS

1. The kit has to be used by skilled and properly trained technical personnel only, under the supervision of a medical doctor responsible of the laboratory.
2. When the kit is used for the screening of blood units and blood components, it has to be used in a laboratory certified and qualified by the national authority in that field (Ministry of Health or similar entity) to carry out this type of analysis.
3. All the personnel involved in performing the assay have to wear protective laboratory clothes, talc-free gloves and glasses. The use of any sharp (needles) or cutting (blades) devices should be avoided. All the personnel involved should be trained in biosafety procedures, as recommended by the Center for Disease Control, Atlanta, U.S. and reported in the National Institute of Health's publication: "Biosafety in Microbiological and Biomedical Laboratories", ed. 1984.
4. All the personnel involved in sample handling should be vaccinated for HBV and HAV, for which vaccines are available, safe and effective.
5. The laboratory environment should be controlled so as to avoid contaminants such as dust or air-born microbial agents, when opening kit vials and microplates and when performing the test. Protect the Chromogen/Substrate from strong light and avoid vibration of the bench surface where the test is undertaken.
6. Upon receipt, store the kit at 2..8°C into a temperature controlled refrigerator or cold room.
7. Do not interchange components between different lots of the kits. It is recommended that components between two kits of the same lot should not be interchanged.
8. Check that the reagents are clear and do not contain visible heavy particles or aggregates. If not, advise the laboratory supervisor to initiate the necessary procedures for kit replacement.
9. Avoid cross-contamination between serum/plasma samples by using disposable tips and changing them after each sample.

10. Avoid cross-contamination between kit reagents by using disposable tips and changing them between the use of each one.
11. Do not use the kit after the expiration date stated on the external container and internal (vials) labels.
12. Treat all specimens as potentially infective. All human serum specimens should be handled at Biosafety Level 2, as recommended by the Center for Disease Control, Atlanta, U.S. in compliance with what reported in the Institutes of Health's publication: "Biosafety in Microbiological and Biomedical Laboratories", ed. 1984.
13. The use of disposable plastic-ware is recommended in the preparation of the liquid components or in transferring components into automated workstations, in order to avoid cross contamination.
14. Waste produced during the use of the kit has to be discarded in compliance with national directives and laws concerning laboratory waste of chemical and biological substances. In particular, liquid waste generated from the washing procedure, from residuals of controls and from samples has to be treated as potentially infective material and inactivated before waste. Suggested procedures of inactivation are treatment with a 10% final concentration of household bleach for 16-18 hrs or heat inactivation by autoclave at 121°C for 20 min..
15. Accidental spills from samples and operations have to be adsorbed with paper tissues soaked with household bleach and then with water. Tissues should then be discarded in proper containers designated for laboratory/hospital waste.
16. The Sulphuric Acid is irritant. In case of spills, wash the surface with plenty of water
17. Other waste materials generated from the use of the kit (example: tips used for samples and controls, used microplates) should be handled as potentially infective and disposed according to national directives and laws concerning laboratory wastes.

G. SPECIMEN: PREPARATION AND RECOMMANDATIONS

1.Blood is drawn aseptically by venepuncture and plasma or serum is prepared using standard techniques of preparation of samples for clinical laboratory analysis. No influence has been observed in the preparation of the sample with citrate, EDTA and heparin.
2. Avoid any addition of preservatives to samples; especially sodium azide as this chemical would affect the enzymatic activity of the conjugate, generating false negative results.
3. Samples have to be clearly identified with codes or names in order to avoid misinterpretation of results. When the kit is used for the screening of blood units, bar code labeling and electronic reading is strongly recommended.
4. Haemolysed (red) and visibly hyperlipemic ("milky") samples have to be discarded as they could generate false results. Samples containing residues of fibrin or heavy particles or microbial filaments and bodies should be discarded as they could give rise to false results.
5. Sera and plasma can be stored at +2°..8°C for up to five days after collection. For longer storage periods, samples can be stored frozen at –20°C for several months. Any frozen samples should not be frozen/thawed more than once as this may generate particles that could affect the test result.
6. If particles are present filter using 0.2-0.8u filters to clean up the sample for testing.
7. Do not use heat inactivated samples as they could give origin to false reactivity.

H. PREPARATION OF COMPONENTS AND WARNINGS

A study conducted on an opened kit has not pointed out any relevant loss of activity up to 2 months.

Microplates:
Allow the microplate to reach room temperature (about 1 hr) before opening the container. Check that the pouch is not

broken or that some defect is present indicating a problem of storage. In this case call Dia.Pro's customer service.

Unused strips have to be placed back into the aluminum pouch, in presence of desiccant supplied, firmly zipped and stored at +2°..8°C. When opened the first time, residual strips are stable up to two months.

Negative Control:

Ready to use. Mix well on vortex before use.

Positive Control:

Ready to use. Mix well on vortex before use. Handle this component as potentially infective, even if a potential infectious agent, if present in the control, has been chemically inactivated.

Wash buffer concentrate:

The 20x concentrated solution has to be diluted with EIA grade water up to 1200 ml and mixed gently end-over-end before use. As some salt crystals may be present into the vial, take care to dissolve all the content when preparing the solution.

In the preparation avoid foaming as the presence of bubbles could give origin to a bad washing efficiency.

Note: Once diluted, the wash solution is stable for 1 week at +2..8° C.

Conjugate # 1:

The Conjugate # 1 mix solution must be prepared immediately before the dispensation of the samples. Add 6 ml Conjugate # 1 diluent directly to one vial of Conjugate # 1 to dissolve the lyophilized powder. This preparation is sufficient for 32 tests, or 4 complete strips.

Important Note: Any unused portion of this reconstituted Conjugate # 1 Solution may be stored at 2...8°C for no more than 12 hours.

Conjugate # 2:

Ready to use reagent. Mix well on vortex before use.

Chromogen/Substrate:

Ready to use. Mix well on vortex before use.

Be careful not to contaminate the liquid with oxidizing chemicals, air-driven dust or microbes. Do not expose to strong illumination, oxidizing agents and metallic surfaces.

If this component has to be transferred use only plastic, possible sterile disposable container.

Sulphuric Acid:

Ready to use. Mix well on vortex before use.

Attention: Irritant (Xi R36/38; S2/26/30)

Legenda: R 36/38 = Irritating to eyes and skin.

S 2/26/30 = In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.

Sample Diluent:

Ready to use. Mix well on vortex before use.

Calibrator:

Dissolve carefully the content of the lyophilized vial with the volume of EIA grade water reported on its label.

Mix well on vortex before use.

I. INSTRUMENTS AND TOOLS USED IN COMBINATION WITH THE KIT

1. **Micropipettes** have to be calibrated to deliver the correct volume required by the assay and must be submitted to regular decontamination (household alcohol, 10% solution of bleach, hospital grade disinfectants) of those parts that could accidentally come in contact with the sample. They should also be regularly maintained in order to show a precision of 1% and a trueness of +/-2%. Decontamination of spills or residues of kit components should also be carried out regularly.

2. The **ELISA incubator** has to be set at +37°C (tolerance of +/-0.5°C) and regularly checked to ensure the correct temperature is maintained. Both dry incubators and water baths are suitable for the incubations, provided that the instrument is validated for the incubation of ELISA tests.

3. The **ELISA washer** is extremely important to the overall performances of the assay. The washer must be carefully validated and correctly optimized using the kit controls and reference panels, before using the kit for routine laboratory tests. Usually 4-5 washing cycles (aspiration + dispensation of 350ul/well of washing solution = 1 cycle) are sufficient to ensure that the assay performs as expected. A soaking time of 20-30 seconds between cycles is suggested. In order to set correctly their number, it is recommended to run an assay with the kit controls and well characterized negative and positive reference samples, and check to match the values reported below in the section "Internal Quality control". Regular calibration of the volumes delivered by, and maintenance (decontamination and cleaning of needles) of the washer has to be carried out according to the instructions of the manufacturer.

4. Incubation times have a tolerance of $\pm5\%$.

5. The **ELISA reader** has to be equipped with a reading filter of 450nm and with a second filter (620-630nm, strongly recommended) for blanking purposes. Its standard performances should be (a) bandwidth $\leq$ 10 nm; (b) absorbance range from 0 to $\geq$ 2.0; (c) linearity to $\geq$ 2.0; repeatability $\geq$ 1%. Blanking is carried out on the well identified in the section "Assay Procedure". The optical system of the reader has to be calibrated regularly to ensure that the correct optical density is measured. It should be regularly maintained according to the manufacturer 's instructions.

6. When using an **ELISA automated work station**, all critical steps (dispensation, incubation, washing, reading, data handling) have to be carefully set, calibrated, controlled and regularly serviced in order to match the values reported in the section "Internal Quality Control". The assay protocol has to be installed in the operating system of the unit and validated as for the washer and the reader. In addition, the liquid handling part of the station (dispensation and washing) has to be validated and correctly set. Particular attention must be paid to avoid carry over by the needles used for dispensing and for washing. This must be studied and controlled to minimize the possibility of contamination of adjacent wells. The use of ELISA automated work stations is recommended for blood screening when the number of samples to be tested exceed 20-30 units per run.

7. When using automatic devices, in case the vial holder of the instrument does not fit with the vials supplied in the kit, transfer the solution into appropriate containers and label them with the same label peeled out from the original vial. This operation is important in order to avoid mismatching contents of vials, when transferring them. When the test is over, return the secondary labeled containers to 2..8°C, firmly capped.

8. Dia.Pro's customer service offers support to the user in the setting and checking of instruments used in combination with the kit, in order to assure compliance with the requirements described. Support is also provided for the installation of new instruments to be used with the kit.

L. PRE ASSAY CONTROLS AND OPERATIONS

1. Check the expiration date of the kit printed on the external label of the kit box. Do not use if expired.

2. Check that the liquid components are not contaminated by naked-eye visible particles or aggregates. Check that the Chromogen/Substrate is colorless or pale blue by aspirating a small volume of it with a sterile transparent plastic pipette. Check that no breakage occurred in transportation and no spillage of liquid is present inside the box. Check that the aluminum pouch, containing the microplate, is not punctured or damaged.

3. Dilute all the content of the 20x concentrated Wash Solution as described above.
4. Dissolve the Conjugate # 1 as described in the proper section.
5. Allow all the other components to reach room temperature (about 1 hr) and then mix as described.
6. Set the ELISA incubator at +37°C and prepare the ELISA washer by priming with the diluted washing solution, according to the manufacturers instructions. Set the right number of washing cycles as found in the validation of the instrument for its use with the kit.
7. Check that the ELISA reader has been turned on at least 20 minutes before reading.
8. If using an automated workstation, turn it on, check settings and be sure to use the right assay protocol.
9. Check that the micropipettes are set to the required volume.
10. Check that all the other equipment is available and ready to use.
11. In case of problems, do not proceed further with the test and advise the supervisor.

M. ASSAY PROCEDURE
The assay has to be carried out according to what reported below, taking care to maintain the same incubation time for all the samples in testing.

Automated assay:
In case the test is carried out automatically with an ELISA system, we suggest to make the instrument dispense 50 ul Sample Diluent first and then 150 ul controls and samples.
Before the next sample is aspirated, needles have to be duly washed to avoid any cross-contamination among samples or tips have to be changed. For the next operations follow the operative instructions reported below for the Manual Assay.
It is strongly recommended to check that the time lap between the dispensation of the first and the last sample will be calculated by the instrument and taken into consideration by delaying the first washing operation accordingly.

Manual assay:
1. Resuspend the content of the correct number of Conjugate # 1 vials with Conjugate # 1 Diluent before starting to dispense samples and controls.
2. Place the required number of wells in the microplate holder. Leave the 1st well empty for the operation of blanking.
3. Dispense 50 ul Sample Diluent in all the wells, except A1 used for blanking.
4. Then dispense 150 ul of Negative Control in triplicate, 150 ul Positive Control in single and then 150 ul of Calibrator in duplicate in proper wells.
5. Add 150 ul of Samples in each properly identified well. Mix gently the plate on the work surface, avoiding overflowing and contaminating adjacent wells, in order to fully disperse the sample into the diluent.
6. Incubate the microplate for **60 min at +37°C**.

Important note: Strips have to be sealed with the adhesive sealing foil, supplied, only when the test is carried out manually. Do not cover strips when using ELISA automatic instruments.

7. Wash the microplate with an automatic washer by delivering and aspirating 350ul/well of diluted washing solution as reported previously (section I.3).
8. Pipette 150 ul Conjugate # 1 mix, prepared as described before, into each well, except the 1st blanking well, and cover with the sealer.
Important note: Be careful not to touch the plastic inner surface of the well with the tip filled with the Conjugate. Contamination might occur.
9. Incubate the microplate for **30 min at +37°C**.
10. Pipette 100 ul of Conjugate # 2 in all the wells, except A1, and gently agitate the microplate to mix the conjugates.

Important Note: This solution must be added to the bottom of each well to ensure proper performance. Inadequate mixing of the two solutions (Conjugate # 1 and Conjugate # 2) may reduce the binding of Streptavidin HRP (Conjugate # 2) to the biotinylated reagents and consequently affect the performance of the assay. Be sure to provide an adequate mixing when the Conjugate # 2 is added, both in the manual and in the automated procedures.

11. Incubate the microplate sealed for **30 min at +37°C**.
12. Wash as in section 7.
13. Dispense 200 ul of Chromogen/Substrate mixture into each well, the blank well included. Then incubate the microplate at **room temperature (18-25°C) for 30 minutes**. Start the timing immediately after addition of this component to the first well.

Important note: Do not expose to strong direct illumination. High background might be generated.

14. Pipette 100 ul Sulphuric Acid into all the wells using the same pipetting sequence as in step 13 to stop the enzymatic reaction. Addition of acid will turn the positive controls and positive samples from blue to yellow.
15. Measure the color intensity of the solution in each well, as described in section I.5, at 450nm filter (reading) and at 620-630nm (background subtraction, strongly recommended), blanking the instrument on A1.

Important notes:
1. *If the second filter is not available ensure that no finger prints are present on the bottom of the microwell before reading at 450nm. Finger prints could generate false positive results on reading.*
2. *Reading has to be carried out just after the addition of the Stop Solution and anyway not any longer than 30 minutes after its addition. Some self oxidation of the chromogen can occur leading to high background.*

N. ASSAY SCHEME

Method	Operations
Sample Diluent	50 ul
Controls	150 ul
Calibrator(*)	150 ul
Samples	150 ul
1st incubation	**60 min**
Temperature	+37°C
Wash step	4-5 cycles
Conjugate # 1	150 ul
2nd incubation	**30 min**
Temperature	+37°C
Conjugate # 2	100 ul
3rd incubation	**30 min**
Temperature	+37°C
Wash step	4-5 cycles
TMB/H2O2	200 ul
4th incubation	**30 min**
Temperature	r.t.
Sulphuric Acid	100 ul
Reading OD	450nm

(*) Important Notes:
- The Calibrator (CAL) does not affect the Cut Off calculation, therefore it does not affect the test's results calculation.
- The Calibrator (CAL) used only if a laboratory internal quality control is required by the Management.

An example of dispensation scheme is reported below:

Microplate

	1	2	3	4	5	6	7	8	9	10	11	12
A	BLK	S2										
B	NC	S3										
C	NC	S4										
D	NC	S5										
E	CAL(*)	S6										
F	CAL (*)	S7										
G	POS	S8										
H	S1	S9										

Legenda: BLK = Blank NC = Negative Control POS = Positive Control S = Sample CAL(*) = Calibrator − Not Mandatory

O. INTERNAL QUALITY CONTROL

A check is carried out on the controls and the calibrator any time the kit is used in order to verify whether their OD450nm values are as expected and reported in the table below.

Check	Requirements
Blank well	≤ 0.100 OD450nm value
Negative Control (NC)	≤ 0.200 OD450nm value after blanking Absorbance of individual negative control values must be less than or equal to 0.200. If one value is outside this range, discard this value and recalculate mean. If two values are outside this range the run should be repeated.
Positive Control	Mean OD450nm ≥ 0.500

If the results of the test match the requirements stated above, proceed to the next section.
If they do not, do not proceed any further and operate as follows:

Problem	Check
Blank well > 0.100 OD450nm	1. that the Chromogen/Sustrate solution has not got contaminated during the assay
Negative Control (NC) > 0.200 OD450nm after blanking	1. that the washing procedure and the washer settings are as validated in the pre qualification study; 2. that the proper washing solution has been used and the washer has been primed with it before use; 3. that no mistake has been done in the assay procedure (dispensation of positive control instead of negative control; 4. that no contamination of the negative control or of their wells has occurred due to positive samples, to spills or to the enzyme conjugate; 5. that micropipettes haven't got contaminated with positive samples or with the enzyme conjugate 6. that the washer needles are not blocked or partially obstructed.
Positive Control < 0.500 OD450nm	1. that the procedure has been correctly executed; 2. that no mistake has been done in the distribution of controls (dispensation of negative control instead of positive control. In this case, the negative control will have an OD450nm value > 0.150, too. 3. that the washing procedure and the washer settings are as validated in the pre qualification study; 4. that no external contamination of the positive control has occurred.

Should these problems happen, after checking, report any residual problem to the supervisor for further actions.

** Note:

If the Calibrator has used, verify the following data:

Check	Requirements
Calibrator	S/Co > 1.0

If the results of the test doesn't match the requirements stated above, operate as follows:

Problem	Check
Calibrator S/Co < 1	1. that the procedure has been correctly executed; 2. that no mistake has been done in its distribution (e.g.: dispensation of negative control instead); 3. that the washing procedure and the washer settings are as validated in the pre qualification study; 4. that no external contamination of the calibrator has occurred.

Anyway, if all other parameters (Blank, Negative Control, Positive Control), match the established requirements, the test may be considered valid.

P. CALCULATION OF THE CUT-OFF

The tests results are calculated by means of a cut-off value determined with the following formula on the mean OD450nm value of the Negative Control (NC):

NC + 0.300 = Cut-Off (Co)

The value found for the test is used for the interpretation of results as described in the next paragraph.

Important note: When the calculation of results is done by the operative system of an ELISA automated work station be sure that the proper formulation is used to calculate the cut-off value and generate the right interpretations of results.

Q. INTERPRETATION OF RESULTS

Test results are interpreted as ratio of the sample OD450nm and the Cut-Off value (or S/Co) according to the following table:

S/Co	Interpretation
< 0.9	Negative
0.9 − 1.1	Indeterminate
> 1.1	Positive

Samples showing a value of S/Co < 0.9 are considered negative and this result indicates that the patient has not been infected by Plasmodium species.
Samples showing a value of S/Co > 1.1 are considered positive and this result is indicative of a recent or past Plasmodium species infection.
Samples showing a S/Co value in the gray-zone 0.9 − 1.1 have to be retested after 2-3 weeks to verify whether or not the result has become positive.

Important notes:
1. Interpretation of results should be done under the supervision of the responsible of the laboratory to reduce the risk of judgment errors and misinterpretations.
2. According to US NIH's directive, any positive result in blood screening should be confirmed by a Confirmatory method capable to detect antibodies to Malaria antigens before a diagnosis of infection is formulated.
3. Nucleic Acid Tests (NATs) for Malaria ssp are not intended to confirm an antibody assay by definition. However they may be used by the responsible of the laboratory to decide whether or not the blood unit can be transfused, even in presence of antibodies (ask DiaPro srl for Malaria ssp RealTime PCR kit).
4. As proved in the Performance Evaluation of the product, the assay is able to detect anti Malaria ssp antibodies **earlier** than some other commercial kit. Therefore a positive result, not confirmed with these less sensitive commercial kits, can not be considered a false positive result, unless other evidences are present. The sample should be submitted to a Confirmation assay.

5. When test results are transmitted from the laboratory to an informatics centre, attention has to be done to avoid erroneous data transfer.
6. Diagnosis of Malaria infection has to be done and released to the patient only by a qualified medical doctor. The presence of antibodies does not mean, anyway, that the patient is undergoing an infection at the moment of analysis. Antibodies can last for the life of the patient even in absence of Malaria ssp live organisms in blood The diagnosis of Malaria ssp infection should be done only in presence of other clinical and diagnostic evidences (presence of Malaria antigen in blood by RealTime PCR or other methods).

An example of calculation is reported below:

The following data must not be used instead or real figures obtained by the user.

Negative Control: 0.048 – 0.050 – 0.052 OD450nm
Mean Value: 0.050 OD450nm
Lower than 0.200 – Accepted
Cut-Off = 0.050+0.300 = 0.350
Positive Control: 1.000 OD450nm mean value
Higher than 0.500 – Accepted
Calibrator: 0.810 OD450nm mean value
S/Co > 1 – Accepted
Sample 1:   0.070 OD450nm
Sample 2:   1.690 OD450nm
Sample 1 S/Co < 1 = negative
Sample 2 S/Co > 1 = positive

R. PERFORMANCES

1. SENSITIVITY:
The Analytical Sensitivity of the assay, in absence of a EC defined international standard, has defined on the sample coded # 71281 (version 3 15 September 2005) of the panel of positive Ab samples to Plasmodium species produced by NIBSC, UK.
Results of limiting dilution of the positive sample into a negative specimen (Dil.) are reported in the table below with reference to a CE marked kit (Diamed/Biorad):

Dil.	Lot # 1		Lot # 2		Lot # 3		Diamed
	OD450	S/Co	OD450	S/Co	OD450	S/Co	S/Co
1x	0.769	2.1	0.905	2.4	0.964	2.8	0.4
2x	0.384	1.1	0.452	1.2	0.481	1.4	0.4
4x	0.174	0.5	0.224	0.6	0.242	0.7	0.3
Dil.	0.054	0.1	0.051	0.1	0.055	0.1	0.4

The Diagnostic Sensitivity of the assay has been calculated on a panel of samples positive for antibodies to Plasmodium species, previously classified positive by a reference method.
The test shows a sensitivity > 95% on plasma and sera.
In addition the sensitivity of the system was also assessed on the panel supplied by NIBSC, UK, for antibodies to Plasmodium species.
Results (S/Co values) for three lots of product are reported in the table below with reference to a CE marked kit (Diamed/Biorad):

Member	Lot # 1 S/Co	Lot # 2 S/Co	Lot # 3 S/Co	Diamed S/Co
72/345	1.8	2.7	2.6	1.6
71/281	2.1	2.4	2.8	0.4
71/326	3.6	1.4	1.6	0.9
72/341	3.6	4.4	4.1	1.6
72/348	3.4	2.6	2.7	1.6
72/138	0.5	0.4	0.5	0.8

2. DIAGNOSTIC SPECIFICITY:
It has been calculated on panels of negative blood donors, previously determined negative by the reference method (Diamed/Biorad).
The assay shows a specificity > 98% on plasma and sera.

3. REPRODUCIBILITY:
It has been evaluate by examining the negative control, the calibrator—and the positive control in 16 replicates in three different runs carried out with the device lot # 0407.
Results are reported in the tables below:

Negative Sample (N = 16)

Mean values	1st run	2nd run	3rd run	Average value
OD 450nm	0.136	0.146	0.153	0.145
Std.Deviation	0.009	0.014	0.011	0.011
CV %	6.4	9.3	7.5	7.7

Calibrator (N = 16)

Mean values	1st run	2nd run	3rd run	Average value
OD 450nm	0.889	0.860	0.844	0.864
Std.Deviation	0.051	0.048	0.094	0.064
CV %	4.3	3.8	7.0	5.0

Positive Sample (N = 16)

Mean values	1st run	2nd run	3rd run	Average
OD 450nm	3.191	3.300	3.175	3.222
Std.Deviation	0.062	0.098	0.103	0.088
CV %	1.9	3.0	3.2	2.7

From the data above the following statistical values have been derived :

Mean values N = 48	Negative Sample	Calibrator	Positive Sample
OD450nm	0.145	0.864	3.222
Std.Deviation	0.011	0.064	0.088
CV %	7.7	5.0	2.7

REFERENCES
1. Garcia LS et al. Update on Malaria. Clin Microbiol News Lett; 1992, 14:65-9
2. Krogstad DJ et al. Plasmodium species (Malaria) principles and practice of infectious diseases, 4th ed, MandellGL et al., 1995, 2415-27.
3. Smith JH et al. Malaria: clinical laboratory features. Clin Microbiol News Lett, 1995,17(24): 185-8.

All the IVD Products manufactured by the company are under the control of a certified Quality Management System in compliance with EN ISO 13485 rule. Each lot is submitted to a quality control and released into the market only if conforming with the EC technical specifications and acceptance criteria.

Produced by
Dia.Pro. Diagnostic Bioprobes Srl.
Via G. Carducci n° 27 – Sesto San Giovanni (Mi) – Italy

CE

PAN MALARIA ANTIBODY CELISA

INTENDED USE AND PRINCIPLE OF THE TEST

The Pan Malaria Antibody CELISA is for the detection of specific IgG antibody against *P. falciparum, P. vivax, P. malariae* and *P. ovale in serum and plasma samples.* The indirect or sandwich ELISA principle is used. Microwells are coated with a panel of recombinant *malaria* antigen. A conjugate of enzyme labelled anti-human globulin is incorporated into the kit. Diluted serum sample is added to the coated wells, which are then incubated to allow antibody to fix to the antigen. Other serum components are then removed by a wash step. The conjugate is then added, binding to any antibody fixed to the well. The well is washed and enzyme substrate solution is added. The amount of colour generated is proportional to the amount of malarial antibodies present in the serum under test.

CONTENTS OF THE KIT

MBCMW	Celisa Plate - 1x 96 wells - (single use only)	2 plates
CONTROL +	Positive Control	0.10 mL
CONTROL -	Negative Control	0.10 mL
MBCPO	Enzyme Conjugate (200x)	0.15 mL
MBCPT	PBS/Tween (20x)	125 mL
MBCSC	Substrate Chromogen (20x)	1.2 mL
MBCSB	Substrate Buffer	24 mL
MBCSS	Stopping Solution	12 mL

Store all components at 2-8°C. Expiry dates are clearly marked on each kit component and on the box. Expiry dates do not change once opened.

MATERIALS REQUIRED BUT NOT PROVIDED

Micropipettes and tips, clean glassware or plastic containers for solutions, distilled water, humid chamber, ELISA washer, Spectrophotometer to read absorbances at a single wavelength of 450nm, or at dual wavelengths of 450nm and 620nm.

PRECAUTIONS

For in vitro diagnostic use only. Reagents should not be used after the expiry date shown on the label. If protective packaging is damaged, contact your local distributor and ask for a replacement. Do not mix reagents from different kits. Thimerosal preservative added to some components is a poison. Exercise caution when handling these components. The stopping solution is corrosive. Avoid contact with skin, eyes and mucous membranes. Dispense all reagents with care to avoid cross contamination of wells. Avoid exposure of the substrate to light. Treat all clinical and control material as though potentially infectious and dispose of in accordance with local operating regulations. For further information, please refer to the Material Safety Data Sheet.

INSTRUCTIONS FOR USE

Preparation of Wash Buffer

If crystals are present in the concentrate, warm to dissolve. For each microplate, add 50mL PBS-Tween concentrate MBCPT to 950mL of distilled water. Label the bottle WASH BUFFER. Store at 2-8°C. Use WASH BUFFER to dilute samples, conjugate concentrate MBCPO and washing the plates.

Preparation of samples

Fresh, refrigerated or frozen samples of serum or plasma may be used. Avoid contamination by collecting aseptically. Prepare the samples by making a 1/100 dilution of the CONTROL +, the CONTROL - and the test (patient specimen) samples in WASH BUFFER, ensuring proper mixing.

Assay Procedure

1. Bring all reagents to room temperature (18-25 °C) before use.
2. Prepare WASH BUFFER (see Preparation of Wash Buffer), diluted CONTROL +, diluted CONTROL - and diluted sample (see Preparation of samples).
3. Remove required number of MBCMW strips. Reseal the foil bag containing unused microwell strips immediately with tape.
4. Pipette 100µL of diluted CONTROL +, diluted CONTROL - and DILUTED SAMPLE into individual microwells. Include two positive and two negative controls in each assay run. Cover and incubate for one (1) hour at room temperature (RT) (18°C – 25°C) in a humid chamber.
5. In the last 10 minutes of the incubation period, prepare the working strength CONJUGATE. Add 5µL of Enzyme Conjugate MBCPO to 995µL of WASH BUFFER and mix thoroughly (allow 1mL per strip of 8 wells).
6. Wash the wells preferably using an automatic plate/strip washer or manually as follows:
   -Empty contents from the wells. Refill with the WASH BUFFER.
   -Repeat this process a further three (3) times. Shake out well contents at the end of the fourth wash.
   -NB: take care when flicking out plates, hold side of frame firmly to hold strips in place.
7. Add 100µL of CONJUGATE to each well. Incubate for one (1) hour at room temperature (RT) in a humid chamber.
8. In the last 10 minutes of the incubation period, prepare the working strength SUBSTRATE. Add 50µL of Substrate Chromogen MBCSC to 950µL of Substrate Buffer MBCSB and mix thoroughly (allow 1 mL per strip of 8 wells). The stability of the solution is 30 minutes.
9. Repeat washing as in step 6.
10. Add 100µL of fresh SUBSTRATE and incubate in the dark (covered) at room temperature for 15 minutes.
11. Add 50µL of Stopping Solution MBCSS. Tap the plate to mix.
12. Read the results visually or in a spectrophotometer at 450nm, or 450nm/620nm, blanking the machine on air.

READING AND INTERPRETATION OF RESULTS AND DIAGNOSIS

Visually

Observe the colour intensity of the control and specimen wells. The Positive Control should be blue before, and yellow after stopping.

Photometrically

Read the microwell plate at 450nm or 450nm / 620nm in a compatible ELISA plate reader, blanked against air. For the test results to be accepted the controls must read as follows:

	O.D Value (450nm)	O.D Value (450/620nm)
Positive Control	>1.500 OD	>1.500 OD
Negative Control	<0.250 OD	<0.200
Cut-Off level (COV)	= Negative Control OD + 0.1	

If controls do not satisfy above criteria, repeat the test.

Negative serum samples should give optical density readings below 0.250 OD units at 450nm or below 0.200 OD units at 450/620nm. However, to allow for inter-laboratory variation we strongly recommend that each laboratory run a number of known negative blood samples to allow standardisation of the CELISA positive / negative cut-off level.

246

Those specimens giving absorbance values below the COV mentioned above are regarded as negative i.e. do not contain amounts of antibody measurable by this test. Those specimens giving absorbance values above the COV may contain antibody and are generally considered to be at or above the significant level. Serum samples that give values above the COV should be considered as positive for malaria antibody. This suggests that the donor has or has had malaria. It does not imply in any sense that the donor is carrying malaria parasites at this particular time.

WASTE DISPOSAL

Dispose of any unused components as biohazardous waste. For more information, please refer to the MSDS.

DATA ON THE PAN MALARIA ANTIBODY CELISA

Refer to summary table at end of insert. All data on the Pan Malaria Antibody CELISA can be obtained in the product information sheet. Please ask your local distributor or contact Cellabs.

INDEMNITY NOTICE

Modifications or changes made in the recommended procedure may affect the stated or implied claims. A positive or negative result does not preclude the presence of other underlying causative agents. Cellabs and its agents and distributors shall not be liable for damages under these circumstances

FIGURE 1 PAN MALARIA DIAGRAM FOR USE

Dilute CONTROL + 1/100 in WASH BUFFER	Dilute test sample 1/100 in WASH BUFFER	Dilute CONTROL − 1/100 in WASH BUFFER
Diluted CONTROL +	DILUTED SAMPLE	Diluted CONTROL −

Add 100µL to individual MBCMW wells

Incubate for 1 hour in a humid chamber at RT

Wash 4x with WASH BUFFER

Add 100µL CONJUGATE to each well

Incubate for 1 hour in a humid chamber at RT

Wash 4x with WASH BUFFER

Add 100µL of SUBSTRATE to each well

Incubate for 15 minutes in the dark at RT

Add 50µL MBCSS to each well

Read visually, at 450nm or 450/620nm

TABLE 1:   SENSITIVITY, SPECIFICITY, & OTHER DATA ON THE PAN MALARIA CELISA
TABLEAU 1:   SENSIBILITE, SPECIFICITE ET AUTRES DONNEES DU TEST PAN MALARIA CELISA
TABELLE 1:   SENSITIVITÄT, SPEZIFITÄT UND ANDERE DATEN ZUM PAN MALARIA CELISA
TABELLA 1:   SENSIBILITA', SPECIFICITA' ED ALTRI DATI SULLA PAN MALARIA CELISA
TABLA 1:   SENSIBILIDAD, ESPECIFICIDAD Y OTROS DATOS DEL PAN MALARIA CELISA
TABELA 1:   SENSIBILIDADE, ESPECIFICIDADE E OUTROS DADOS DO PAN MALARIA CELISA

Trial	Sensitivity	Specificity	Repeatability	Reproducibility
Essai	Sensibilité	Spécificité	Répétabilité	Reproductibilité
Versuch	Sensitivität	Spezifität	Wiederholpräzision	Reproduzierbarkeit
Prova	Sensibilita'	Specificita'	Ripetibilita	Riproducibilita
Prueba	Sensibilidad	Especificidad	Repetibilidad	Reproducibilidad
Teste	Sensibilidade	Especificidade	Repetição	Reprodutibilidade
A	94%	100%	-	-
B	-	-	Positive CV = 2.75%	Positive CV = 5.81%
	Not cross reactive with / Pas de Réaction Croisée avec / Keine Kreuzreaktionen mit / Non mostra reazione crociata con / No muestra reacción cruzada con / Não apresenta reacções cruzadas com: Toxocara sp., *T. cruzi*, Leishmania sp., *W. bancrofti*, Dengue virus.			

EXPLANATION OF SYMBOLS

[i]	Consult Instructions for Use	▲	Cellabs Pty Ltd Unit 7, 27 Dale Street (PO Box 421) Brookvale, NSW 2100 Australia Tel: +61 2 9905 0133 Fax: +61 2 9905 6426 Web: http://www.cellabs.com.au Email: sales@cellabs.com.au
IVD	*In Vitro* Diagnostic Medical Device		
2°C / 8°C	Temperature Limitation		
LOT	Batch	EC REP	WMDE Bergerweg 18 6085 AT Horn The Netherlands
CONTROL +	Control Positive		
CONTROL −	Control Negative	Insert Version	en fr de it es pt LMC3.20 24 August 2014
⧗	Use By/Expiration Date	CE	0843
⊗	Do Not Re-use		

NovaLisa ™

Malaria

ELISA CE

Enzyme immunoassay for the qualitative determination of antibodies against Plasmodium in human serum or plasma

Enzymimmunoassay zur qualitativen immunenzymatischen Bestimmung von Antikörpern gegen Plasmodium in Humanserum oder Plasma

Enzyme immunoassay pour la détermination qualitative des anticorps contre Plasmodium en sérum humain ou plasma

Test immunoenzimatico per la determinazione qualitativa degli anticorpi per Plasmodium nel siero o plasma umano

Enzimoinmunoensayo para la determinación cualitativa de anticuerpos contra – Plasmodium en suero o plasma humano

Imunoensaio enzimático para a determinação qualitativa de anticorpos contra Plasmodium em soro ou plasma humano

Only for in-vitro diagnostic use

English:	Page	2 to 6
Deutsch:	Seite	7 bis 11
Francais:	Page	12 à 16
Italiano:	da Pagina	17 a
Espanol:	Página	a
Português:	Página	a

For further languages please contact our authorized distributors.

Bibliography / Literatur / Bibliographie / Bibliografia / Bibliografía / Bibliografia	Page / Seite / Page / Pagina / Página/	14
Symbols Key / Symbolschlüssel / Explication des symboles / Legenda / Símbolos / Legenda dos Símbolos	Page / Seite / Page / Pagina / Página	15
Summary of Test Procedure/ Kurzanleitung Testdurchführung/ Résumé de la procedure de test/ Schema della procedura/ Resumen de la técnica / Resumo do Procedimento de Teste	Page / Seite / Page/ Pagina / Página	16

Product Number: MAL0620 (96 Determinations)

ENGLISH

$\mathsf{C}\mathsf{E}$

1. INTRODUCTION

Malaria is a life-threatening disease which is caused by the protozoon Plasmodium spp. The transmission is mediated by the Anopheles mosquito, but can occur via blood transfusion also. Humans can be infected by four different species of Plasmodium: P. falciparum, P. vivax, P. ovale and P. malariae. Infections with P. falciparum can be deadly. P. falciparum and P. vivax are the most common types. The disease occurs mainly in tropical and subtropical areas.
The Malaria infection induces the production of specific antibodies. In general they can be detected within some days after the occurrence of the parasites in the blood. The concentration of the specific antibodies is proportional to the intensity and duration of infection. The detection of antibodies is more sensitive than the direct detection of the pathogen and independent of the status of the infection. In humans who are infected for the first time the level of the specific antibodies decreases fast after recuperation. In contrast the antibody level decreases slowly (within 2 – 3 years) in re-infected persons who move into non-endemic areas.
The NovaLisa™ Malaria antibody assay is a fast and sensitive enzyme immunoassay for the detection of specific IgG and IgM antibodies against Plasmodium spp.
The microplate is coated with recombinant antigens of P. falciparum and P. vivax. P. ovale and P. malaria are also detected due to the antigenic similarity between the different Plasmodium species.

2. INTENDED USE

The Malaria ELISA is intended for the qualitative determination of antibodies against Plasmodium in human serum or plasma (citrate).

3. PRINCIPLE OF THE ASSAY

The qualitative immunoenzymatic determination of antibodies against Plasmodium is based on the ELISA (Enzyme-linked Immunosorbent Assay) technique.
Microtiter strip wells are precoated with Plasmodium antigens to bind corresponding antibodies of the specimen. After washing the wells to remove all unbound sample material horseradish peroxidase (HRP) labelled anti-human IgG and IgM conjugate is added. This conjugate binds to the captured Plasmodium -specific antibodies. The immune complex formed by the bound conjugate is visualized by adding Tetramethylbenzidine (TMB) substrate which gives a blue reaction product. The intensity of this product is proportional to the amount of Plasmodium -specific antibodies in the specimen. Sulphuric acid is added to stop the reaction. This produces a yellow endpoint colour. Absorbance at 450 nm is read using an ELISA microwell plate reader.

4. MATERIALS

4.1. Reagents supplied

- **Malaria Coated Wells:** 12 breakapart 8-well snap-off strips coated with recombinant Plasmodium antigens (P. falciparum, P. vivax); in resealable aluminium foil.
- **Sample Diluent ***:** 1 bottle containing 100 ml of buffer for sample dilution; pH 7.2 ± 0.2; coloured yellow; ready to use; white cap.
- **Stop Solution:** 1 bottle containing 15 ml sulphuric acid, 0.2 mol/l; ready to use; red cap.
- **Washing Solution (20x conc.)*:** 1 bottle containing 50 ml of a 20-fold concentrated buffer (pH 7.2 ± 0.2) for washing the wells; white cap.
- **Malaria Conjugate**:** 1 bottle containing 20 ml of peroxidase labelled antibody to human IgG and IgM; coloured blue, ready to use; black cap.
- **TMB Substrate Solution:** 1 bottle containing 15 ml 3,3',5,5'-tetramethylbenzidine (TMB); ready to use; yellow cap.
- **Malaria Positive Control***:** 1 bottle containing 2 ml; coloured yellow; ready to use; red cap.
- **Malaria Cut-off Control***:** 1 bottle containing 3 ml; coloured yellow; ready to use; green cap.
- **Malaria Negative Control***:** 1 bottle containing 2 ml; coloured yellow; ready to use; blue cap.

*   contains 0.1 % Bronidox L after dilution
**   contains 0.2 % Bronidox L
***   contains 0.1 % Kathon

4.2. Materials supplied

- 1 Strip holder
- 1 Cover foil
- 1 Test protocol
- 1 distribution and identification plan

2

4.3. Materials and Equipment needed

- ELISA microwell plate reader, equipped for the measurement of absorbance at 450/620nm
- Incubator 37°C
- Manual or automatic equipment for rinsing wells
- Pipettes to deliver volumes between 10 and 1000 µl
- Vortex tube mixer
- Deionised or (freshly) distilled water
- Disposable tubes
- Pipe stand
- Timer

5. STABILITY AND STORAGE

The reagents are stable up to the expiry date stated on the label when stored at 2...8 °C.

6. REAGENT PREPARATION

It is very important to bring all reagents, samples and controls to room temperature (20…25°C) before starting the test run!

6.1. Coated snap-off strips

The ready to use breakapart snap-off strips are coated with recombinant Plasmodium antigens. Store at 2...8°C. *Immediately after removal of strips, the remaining strips should be resealed in the aluminium foil along with the desiccant supplied and stored at 2...8 °; stability until expiry date. After first opening stability until expiry date when stored at 2…8°C.*

6.2. Malaria Conjugate

The bottle contains 20ml of a solution with anti-human-IgG and anti-human IgM horseradish peroxidase, buffer, stabilizers, preservatives and an inert blue dye. The solution is ready to use. Store at 2...8°C. *After first opening stability until expiry date when stored at 2…8°C.*

6.3. Controls

The bottles labelled with Positive, Cut-off and Negative Control contain a ready to use control solution. It contains 0.1% Kathon and has to be stored at 2...8°C. *After first opening stability until expiry date when stored at 2…8°C.*

6.4. Sample Diluent

The bottle contains 100ml phosphate buffer, stabilizers, preservatives and an inert yellow dye. It is used for the dilution of the patient specimen. This ready to use solution has to be stored at 2...8°C. *After first opening stability until expiry date when stored at 2…8°C.*

6.5. Washing Solution (20xconc.)

The bottle contains 50 ml of a concentrated buffer, detergents and preservatives. Dilute Washing Solution 1+19; e.g. 10 ml Washing Solution + 190 ml fresh and germ free redistilled water. The diluted buffer is stable for 5 days at room temperature. *After first opening stability until expiry date when stored at 2…8°C.*

6.6. TMB Substrate Solution

The bottle contains 15 ml of a tetramethylbenzidine/hydrogen peroxide system. The reagent is ready to use and has to be stored at 2...8°C, away from the light. *The solution should be colourless or could have a slight blue tinge. If the substrate turns into blue, it may have become contaminated and should be thrown away. After first opening stability until expiry date when stored at 2…8°C.*

6.7. Stop Solution

The bottle contains 15 ml 0.2 M sulphuric acid solution (R 36/38, S 26). This ready to use solution has to be stored at 2...8°C.
After first opening stability until expiry date.

7. SPECIMEN COLLECTION AND PREPARATION

Use human serum or plasma (citrate) samples with this assay. If the assay is performed within 5 days after sample collection, the specimen should be kept at 2...8°C; otherwise they should be aliquoted and stored deep-frozen (-20 to -70°C). If samples are stored frozen, mix thawed samples well before testing. *Avoid repeated freezing and thawing.*
Heat inactivation of samples is not recommended.

7.1. Sample Dilution

Before assaying, all samples should be diluted 1+100 with Sample Diluent. Dispense 10µl sample and 1ml Sample Diluent into tubes to obtain a 1+100 dilution and thoroughly mix with a Vortex.
Controls are ready to use.

3

8. ASSAY PROCEDURE

8.1. Test Preparation

Please read the test protocol carefully **before** performing the assay. Result reliability depends on strict adherence to the test protocol as described. If performing the test on ELISA automatic systems we recommend to increase the washing steps from three to five and the volume of washing solution from 300µl to 350µl to avoid washing effects. Pay attention to chapter 12. Prior to commencing the assay, the distribution and identification plan for all specimens and controls should be carefully established on the result sheet supplied in the kit. Select the required number of microtiter strips or wells and insert them into the holder.

Please allocate at least:

1 well	(e.g. A1)	for the substrate blank,
1 well	(e.g. B1)	for the negative control,
2 wells	(e.g. C1+D1)	for the cut-off control and
1 well	(e.g. E1)	for the positive control.

It is recommended to determine controls and patient samples in duplicate, if necessary.

Perform all assay steps in the order given and without any appreciable delays between the steps.

A clean, disposable tip should be used for dispensing each control and sample.

Adjust the incubator to 37° ± 1°C.

1. Dispense 100µl controls and diluted samples into their respective wells. Leave well A1 for substrate blank.

2. Cover wells with the foil supplied in the kit.

3. **Incubate for 1 hour ± 5 min at 37±1°C.**

4. When incubation has been completed, remove the foil, aspirate the content of the wells and wash each well three times with 300µl of Washing Solution. Avoid overflows from the reaction wells. The soak time between each wash cycle should be >5sec. At the end carefully remove remaining fluid by tapping strips on tissue paper prior to the next step!

   Note: Washing is critical! Insufficient washing results in poor precision and falsely elevated absorbance values.

5. Dispense 100µl Malaria Conjugate into all wells except for the blank well (e.g. A1). Cover with foil.

6. **Incubate for 30 min at room temperature.** *Do not expose to direct sunlight.*

7. Repeat step 4.

8. Dispense 100µl TMB Substrate Solution into all wells

9. **Incubate for exactly 15 min at room temperature in the dark.**

10. Dispense 100µl Stop Solution into all wells in the same order and at the same rate as for the TMB Substrate Solution.
    Any blue colour developed during the incubation turns into yellow.

    Note: *Highly positive patient samples can cause dark precipitates of the chromogen! These precipitates have an influence when reading the optical density. Predilution of the sample with physiological sodium chloride solution, for example 1+1, is recommended. Then dilute the sample 1+100 with dilution buffer and multiply the results in NTU by 2.*

11. Measure the absorbance of the specimen at 450/620nm within 30 min after addition of the Stop Solution.

8.2. Measurement

Adjust the ELISA Microwell Plate Reader **to zero** using the **substrate blank in well A1**.

If - due to technical reasons - the ELISA reader cannot be adjusted to zero using the substrate blank in well A1, subtract the absorbance value of well A1 from all other absorbance values measured in order to obtain reliable results!

Measure the absorbance of all wells at **450 nm** and record the absorbance values for each control and patient sample in the distribution and identification plan.

Dual wavelength reading using 620 nm as reference wavelength is recommended.

Where applicable calculate the **mean absorbance values** of all duplicates.

9. RESULTS

9.1. Run Validation Criteria

In order for an assay to be considered valid, the following criteria must be met:

- **Substrate blank**   in A1:   Absorbance value **< 0.100.**
- **Negative control**   in B1:   Absorbance value **< 0.200 and < cut-off**
- **Cut-off control**   in C1 and D1:   Absorbance value **0.150 – 1.30.**
- **Positive control**   in E1:   Absorbance value **> cut-off**.

If these criteria are not met, the test is not valid and must be repeated.

4

252

9.2. Calculation of Results

The cut-off is the mean absorbance value of the Cut-off control determinations.

Example: Absorbance value Cut-off control 0.39 + absorbance value Cut-off control 0.37 =0.76 / 2 = 0.38

Cut-off = 0.38

9.3. Interpretation of Results

Samples are considered **POSITIVE** if the absorbance value is higher than 10% over the cut-off.

Samples with an absorbance value of 10% above or below the cut-off should not be considered as clearly positive or negative
→ **grey zone**
It is recommended to repeat the test again 2 - 4 weeks later with a fresh sample. If results in the second test are again in the grey zone the sample has to be considered **NEGATIVE**.

Samples are considered **NEGATIVE** if the absorbance value is lower than 10% below the cut-off.

9.3.1. Results in Units

$$\frac{\text{Patient (mean) absorbance value x 10}}{\text{Cut-off}} = [\text{Units} = \text{NTU}]$$

Example: $\frac{1.786 \times 10}{0.38} = 47\ NTU\ (Units)$

Cut-off:	10	NTU
Grey zone:	9-11	NTU
Negative:	<9	NTU
Positive:	>11	NTU

10. SPECIFIC PERFORMANCE CHARACTERISTICS

10.1. Precision

Interassay	n	Mean (NTU)	Cv (%)
Positive Sample	24	33.6	3.2
Positive Sample	24	30.5	4.8
Negative Sample	24	2.5	10.3

Intraassay	n	Mean (OD)	Cv (%)
Positive Sample	22	1.61	2.8
Positive Sample	22	1.89	3.9
Negative Sample	22	0.20	5.5

10.2. Diagnostic Specificity

The diagnostic specificity is defined as the probability of the assay of scoring negative in the absence of the specific analyte.
It is 97.5 %.

10.3. Diagnostic Sensitivity

The diagnostic sensitivity is defined as the probability of the assay of scoring positive in the presence of the specific analyte.
It is 95.9%.

10.4. Interferences

Interferences with hemolytic, lipemic or icteric sera are not observed up to a concentration of 10 mg/ml hemoglobin, 5 mg/ml triglycerides and 0.5 mg/ml bilirubin.

Note: The results refer to the groups of samples investigated; these are not guaranteed specifications.

11. LIMITATIONS OF THE PROCEDURE

Bacterial contamination or repeated freeze-thaw cycles of the specimen may affect the absorbance values. Diagnosis of an infectious disease should not be established on the basis of a single test result. A precise diagnosis should take into consideration clinical history, symptomatology as well as serological data.
In immunocompromised patients and newborns serological data only have restricted value.
A cross reactivity with antibodies against Trypanosoma, Leishmania, Schistosoma and Toxoplasma cannot be excluded.

5

12. PRECAUTIONS AND WARNINGS

- In compliance with article 1 paragraph 2b European directive 98/79/EC the use of the in vitro diagnostic medical devices is intended by the manufacturer to secure suitability, performances and safety of the product. Therefore the test procedure, the information, the precautions and warnings in the instructions for use have to be strictly followed. The use of the testkits with analyzers and similar equipment has to be validated. Any change in design, composition and test procedure as well as for any use in combination with other products not approved by the manufacturer is not authorized; the user himself is responsible for such changes. The manufacturer is not liable for false results and incidents for these reasons. The manufacturer is not liable for any results by visual analysis of the patient samples.
- Only for in-vitro diagnostic use.
- All components of human origin used for the production of these reagents have been tested for anti-HIV antibodies, anti-HCV antibodies and HBsAg and have been found to be non-reactive. Nevertheless, all materials should still be regarded and handled as potentially infectious.
- Do not interchange reagents or strips of different production lots.
- No reagents of other manufacturers should be used along with reagents of this test kit.
- Do not use reagents after expiry date stated on the label.
- Use only clean pipette tips, dispensers, and lab ware.
- Do not interchange screw caps of reagent vials to avoid cross-contamination.
- Close reagent vials tightly immediately after use to avoid evaporation and microbial contamination.
- After first opening and subsequent storage check conjugate and control vials for microbial contamination prior to further use.
- To avoid cross-contamination and falsely elevated results pipette patient samples and dispense conjugate without splashing accurately to the bottom of wells.
- The ELISA is only designed for qualified personnel who are familiar with good laboratory practice.

WARNING:	In the used concentration Bronidox L has hardly any toxicological risk upon contact with skin and mucous membranes!
WARNING:	Sulphuric acid irritates eyes and skin. Keep out of the reach of children. Upon contact with the eyes, rinse thoroughly with water and consult a doctor!

12.1. Disposal Considerations

Residues of chemicals and preparations are generally considered as hazardous waste. The disposal of this kind of waste is regulated through national and regional laws and regulations. Contact your local authorities or waste management companies which will give advice on how to dispose hazardous waste.

13. ORDERING INFORMATION

Prod. No.:   MAL0620   Malaria ELISA (96 Determinations)

SCHEME OF THE ASSAY

Malaria ELISA

Test preparation

Prepare reagents and samples as described.
Establish the distribution and identification plan for all specimens and controls on the
result sheet supplied in the kit.
Select the required number of microtiter strips or wells and insert them into the holder.

Assay procedure

	Substrate blank (e.g. A1)	Negative control	Positive control	Cut-off control	Sample (diluted 1+100)
Negative control	-	100µl	-	-	-
Positive control	-	-	100µl	-	-
Cut-off control	-	-	-	100µl	-
Sample (diluted 1+100)	-	-	-	-	100µl
Cover wells with foil supplied in the kit **Incubate for 1 h at 37°C** Wash each well three times with 300µl of washing solution					
Conjugate	-	100µl	100µl	100µl	100µl
Cover wells with foil supplied in the kit **Incubate for 30 min at room temperature** Wash each well three times with 300µl of washing solution					
TMB Substrate	100µl	100µl	100µl	100µl	100µl
Incubate for exactly 15 min at room temperature in the dark					
Stop Solution	100µl	100µl	100µl	100µl	100µl
Photometric measurement at 450 nm (reference wavelength: 620 nm)					

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Technologie & Waldpark

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Internet: www.NovaTec-ID.com   MAL0620engl,dt,fr,it-07122012-CR

Appendix D: Standard Operating Procedure for the Research-based

Combined Antigen Enzyme-linked Immunosorbent Assay

SOP Title:	Combined antigen ELISA for sera using TMB	LONDON SCHOOL of HYGIENE &TROPICAL MEDICINE
SOP No:	Combined antigen ELISA TMB	
Version:	002	

Effective from:	13/02/2018
Superseded Version Number:	001

Author:

Name: Tom Hall
Position: Scientific Officer
Signature: Date:

Revision History

Comments

Reviewed by:

Date:

Next review due:

Signature(s):

Combined Sera ELISA TMB v02

1 Overview

This SOP describes the ELISA process in order to estimate levels of specific antibodies to malaria antigens combined into one assay and using TMB.

2 Safety

Adhere to local safety regulations. Wear appropriate personal protective equipment.

3 Materials

Laboratory facilities/Equipment

   a) 96 well ELISA plates: Immulon 4 HBX Flat bottom microtiter plates (Thermo Scientific)

   b) 96 deep well plates (Costar 0.5ml v bottom assay block)

   c) 3 plastic buckets/containers for washing plates

   d) Plate Reader- that can read 450nm

   e) Protective latex or nitrile gloves, safety glasses, lab coat

   f) Multi channel pipettes (8 or 12 channel 2-10ul, 5-50 µl, 30-300 µl)

   g) Range of single channel pipettes

   h) 10ul, 200ul and 100ul pipette tips

   i) Scales, magnetic stirrer, pH meter, vortex#

   j) Distilled water, sink, fridge/freezer

Documents

Laboratory Record Book

Reagents

All reagents should be stored according to the instructions supplied with them and disposed of at the expiry date recorded on the product.

   a) Antigens (PfAMA1, PfMSP119, MSP2 Dd2, MSP2 CH150)

   b) Controls (standard dilutions)

   c) TMB one component HRP microwell substrate (#TMBW-1000-01; Tebu-bio laboratories)

   d) Tween 20 (Sigma)

   e) Skimmed milk powder (Supermarket)

Page **2** of **8**

f) Horseradish peroxidase-conjugated rabbit anti-human IgG (#P0214; Dako)

g) NaH_2PO_4 (Sodium dihydrogen orthophosphate) (VWR International Ltd)

h) Na_2HPO_4 (di-sodium hydrogen orthophosphate) (VWR International Ltd)

i) NaCl (Sodium chloride) (Fisher scientific)

j) H_2SO_4 (Sulphuric acid) (BDH)

k) Na_2CO_3 (Sodium carbonate) (Sigma)

l) $NaHCO_3$ (Sodium hydrogen carbonate) (VWR International Ltd)

4   Preparation of Buffer Solutions

All buffer solutions should be clearly labelled with:

* Reagent name

* Expiry date

* Preparation date

* Name of person who prepared the buffer

Reagents should be stored under appropriate conditions. See Table 1 for details on preparation and storage.

Table 1: Buffer solutions - preparation

Buffer solution	Reagent/chemical	Amount for 10x PBS	Amount for 20x PBS
Phosphate buffered saline (PBS) stock solution pH 7.2	NaH_2PO_4	5.7 g	11.4 g
	Na_2HPO_4	16.7 g	33.4 g
	NaCl	85 g	170 g
	Distilled water	Make up to 1L	Make up to 1L

Store at room temperature, dispose of after one month

1X PBS/Tween (0.05%) wash solution (5 L)	PBS 10X/ PBS 20X	500 ml / 250ml
	Tween 20	2.5 ml
	Distilled water	4.5 L / 4.75L

Make up as needed daily, dispose of unused solution at the end of each day

Coating buffer pH 9.4-9.6	Na_2CO_3	1.59 g
	$NaHCO_3$	2.93 g
	Distilled water	Make up to 1 L

Store at 4°C, dispose of after one month, **pH should be 9.5 ± 0.2**

BLOCKING SOLUTION: 1% skimmed milk powder in 1X PBS/Tween	Skimmed milk powder	10 g
	1X PBS/Tween	1 L

Make up as needed daily, dispose of unused solution at the end of each day

TMB	Enough for 100ul per well	

Aliquot in to bottle covered in silver foil, leave at RT for 30 mins before use.

0.2 M H_2SO_4	H_2SO_4 concentrate	10.7 ml
	Distilled water	Add concentrate to ~800 ml of water, then top up to 1 L

N.B. Appropriate safety regulations must be adhered to when handling H_2SO_4
Store at room temperature, dispose of after six months

Page **4** of **8**

N.B. Fully dissolve the reagent/chemical prior to use

5   Methods

Plate washing

a) Bucket method-three buckets (1, 2, 3) Half fill each bucket with PBS/Tween wash solution (keep refilling during the process) Expel the contents of the plate into sink/waste container, plunge into bucket 1, discard wash into sink/waste container. Repeat the process again using bucket 2 & then for bucket 3.*

b) Automatic plate washer Program to do a 3 wash OR 5 wash using PBS/Tween wash solution

Drying plates

a) Expel any excess liquid from the wash process on paper towel, but DO NOT let the plates dry out.

* For three times wash, after coating or blocking, wash once each in buckets 1, 2, 3
* For five time wash, after adding samples or conjugate, wash once in bucket 1 and twice in bucket 2 and then 3.

5.1.   ELISA

Day 1

5.1.1.   Mark out ELISA plates. 2 plates are required for each deep well. Label plates 1A, 1B, 2A, 2B etc. A and B denote the 2 parts of each deep well. The A plate containing samples tested in duplicate from columns 1-5 and the B plate the samples from columns 6-10.

5.1.2.   Prepare the antigen coating buffer, taking into account the number of plates requiring coating and ensuring the antigens are diluted to the correct concentration (information about the dilution of each antigen can be found on the antigen tube).

5.1.3.   Add 50μl of this diluted antigen, prepared in 5.1.2, to all wells of the 96 well ELISA plate. Tap the plate to ensure the liquid covers the base of the wells.

5.1.4.   Incubate plates overnight at 4°C, to prevent evaporation cover plates on the top and bottom of your test plate stack. Consider wrapping plates in cling film if evaporation is still a problem.

Day 2

5.1.5.   Wash ELISA plates three times in PBS/Tween wash solution, following the technique described in the methods.

5.1.6.   Dry each plate using method above, add 150µl of 1% blocking solution to each well on the plate.

5.1.7.   Incubate at room temperature for three hours.

5.1.8.   One hour before the end of this time remove an aliquot of the positive control from freezer and allow to thaw. Additionally, remove the sample deep wells from the fridge and mix by placing the deep wells on a rotating platform

5.1.9.   Make up standard dilutions in a deep well plate, refer to **Appendix 1**, ensuring sufficient volume for each plate being assayed

5.1.10.   Wash ELISA plates three times in PBS/Tween wash solution.

5.1.11.   Dry plates. Add 40ul of blocking solution to every well on the ELISA plate.

5.1.12.   To achieve a final sample dilution of 1/1000 add 10ul of samples to the plates (Assuming samples are diluted at 1/200). Follow the layout in table 1 and as is described below (sections 5.1.13-5.1.14).

5.1.13.   Add 10ul positive controls and blanks to columns A11-F11 and A12-F12 to every ELISA plate. These are transferred from the previously prepared stock (Step 5.1.9). The amount prepared depends on number of ELISA plates.

5.1.14.   Add samples. From each column of wells in the sample deep wells transfer the samples into duplicate columns of the ELISA plate, starting with A1 to H1 and A2 to H2. Columns 1-5 of the sample deep-well will be on the first ELISA plate & columns 6-10 will be on the second ELISA plate.

Table 1: ELISA plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample 1		Sample 9		Sample 17		Sample 25		Sample 33			
B	Sample 2		Sample 10		Sample 18		Sample 26		Sample 34			
C	Sample 3		Sample 11		Sample 19		Sample 27		Sample 35		Positive control standard curve	
D	Sample 4		Sample 12		Sample 20		Sample 28		Sample 36			
E	Sample 5		Sample 13		Sample 21		Sample 29		Sample 37			
F	Sample 6		Sample 14		Sample 22		Sample 30		Sample 38			
G	Sample 7		Sample 15		Sample 23		Sample 31		Sample 39		Blank	
H	Sample 8		Sample 16		Sample 24		Sample 32		Sample 40		Blank	

5.1.15.   Incubate plates overnight at 4°C, to prevent evaporation place cover plates on the top and bottom of your test plate stack. Consider wrapping plates in cling film if evaporation is still a problem.

Day 3

5.1.16.   Wash five times in PBS/Tween wash solution. Dry plates.

5.1.17.   Make the appropriate volume for the number of ELISA plates of conjugate solution Preparation: horseradish peroxidase-conjugated rabbit anti-human IgG diluted at **1/15000**, in PBS/Tween wash solution.

5.1.18.   Add 50 µl of conjugate solution to each well of the ELISA plates.

5.1.19.   Incubate for three hours at room temperature.

5.1.20.   Wash five times in PBS/Tween wash solution.

5.1.21.   An hour before required measure out the required amount of TMB into a bottle covered in tinfoil. Leave at room temperature in the dark.

5.1.22.   Add 100 µl per well of TMB substrate solution. Leave at room temperature in the dark for 15 minutes for the assay to develop.

5.1.23.   Stop the reaction by adding 50 µl 0.2 M sulphuric acid (H_2SO_4).

5.1.24.   Read plates as soon as possible at 450 nm and save data.

Appendix 1

Working out positive control serial dilutions:

Rather than prepare controls separately, a stock of positive controls that will be enough for all the ELISA plates is made. This cuts down on variability from separate dilutions. may vary for other antigens.

These dilutions are made up in a deep-well plate. Six, six fold serial dilutions are prepared at a starting dilution of 1/10:

Serial dilution: 1/10, 1/60, 1/360, 1/2160, 1/12960, 1/77760. This is the stock of positive control.

To work out how much control you need, you can follow these equations:

Total control volume needed for each plate- 20µl

No. of plates= n

Blocking solution in wells b,c,d,e,f (A) = 20µl * n + 40µl (for excess)

Transfer volume between wells (B) = a/5 (this is for a 1/6 serial dilution)

Total volume in top well (c)= A + B

Positive control serum/plasma 1/10 dilution in top well (D)= c/10

Blocking solution in top well- C – D

Final dilution of positive control in ELISA plate:

1 in 5 dilution of the stock control onto each ELISA plate (40µl blocking solution & 10µl stock control)

Final serial dilution: 1/50, 1/200, 1/800, 1/3200, 1/12800 & 1/51200

Appendix E: Posters Presenting Results from the Malaria Zero Project for Scientific Conferences Visited During my PhD programme

1. Keystone Symposia. Malaria: From Innovation to Eradication. Kampala, Uganda, 19-23 February 2017. (Attended). Page 266.

2. American Society of Tropical Medicine & Hygiene. Annual Meeting 2017. Baltimore, Maryland, USA, 5-9 Nov 2017. (Not attended – poster presented by Eric Rogier, PhD). Page 267.

3. Multilateral Initiative on Malaria (MIM). 7th MIM Pan African Malaria Conference. Dakar, Senegal, 15-20 April 2018. (Attended). Page 268.

4. American Society of Tropical Medicine & Hygiene. Annual Meeting 2018. New Orleans, Louisiana, USA, 28 Oct-1 Nov 2018. (Attended). Page 269.

The use of antimalarial antibody measurements to assist elimination activities in Haiti

L.L. van den Hoogen[1], E. Rogier[2], G. Stresman[1], T. Druetz[3], T. Eisele[3], R. Ashton[3], A. Existe[4], J. Boncy[4], M. Chang[2], J.F. Lemoine[5], K. Tetteh[1] & C. Drakeley[1] for the **Malaria Zero Consortium**

[1]London School of Hygiene & Tropical Medicine, UK; [2]Centres for Disease Control and Prevention, USA; [3]Tulane University School of Public Health & Tropical Medicine, USA; [4]Laboratoire National de Santé Publique, Haiti; [5]Ministry of Public Health & Population, Haiti

Introduction

- Haiti is considered suitable for elimination with low *Plasmodium falciparum* prevalence, parasites sensitive to chloroquine, a weak vector and low risk of importation.[1,2]

- Measuring and monitoring malaria is challenging in elimination settings. Antimalarial antibody measurements are a unique metric as they reflect historical and recent exposure to malaria.

- We performed exploratory analyses of multi-antigenic responses in relation to malaria exposure in southern Haiti.

Methods

- Filter-paper blood spots from easy-access-groups (schools and churches) were collected in two settings in southern Haiti (Fig. 1).[1] Participants were tested with highly-sensitive (HS-) RDTs and regular RDTs.

- We screened 21 *P. falciparum* antigens (18 blood-stage, 1 liver-stage and 1 sporozoite-stage) using a bead-based assay.[3] Thresholds for antibody positivity were calculated using a finite mixture model.
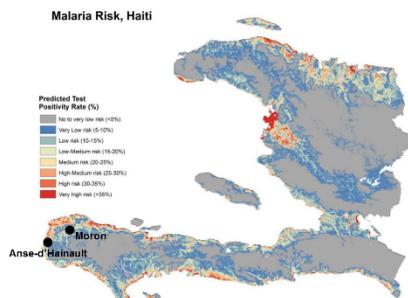

Fig. 1 Predicted malaria risk in Haiti and the two included study sites in the south[1]

Results

- A total of 383 samples were tested. Fifteen individuals were positive by RDT; these were mostly found in the coastal setting Anse-d'Hainault (Table 1).

	Anse-d'Hainault (n/N)	Moron (n/N)
Median age (IQR)	24 (16 - 54)	11 (8-42)
Male	39% (51/131)	12% (31/252)
Venue		
- School	NA	47% (119/252)
- Church	100% (131/131)	53% (133/252)
RDT		
- Negative	90.8% (118/130)	98.8% (235/238)
- HS-RDT only	2.3% (3/130)	0.4% (1/238)
- RDT only	0.0% (0/130)	0.4% (1/238)
- Both RDTs	6.9% (9/130)	0.4% (1/238)

Table 1 General characteristics of the study population in Anse-d'Hainault & Moron, southern Haiti

- Responses to at least 5 antigens showed higher accuracy in detecting any RDT positives (70%; sensitivity 80% and specificity 69%) than responses to the single merozoite antigens AMA1 and/or MSP1$_{19}$ (50%; sensitivity 87% and specificity 49%).
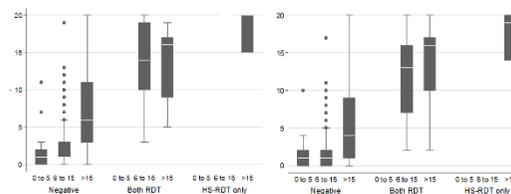

Fig. 2 Breadth of antibody response (a) and frequency of being in the top quintile (b) by RDT status.

- Antibody levels of randomly selected individuals are shown in heat maps (Fig. 3): for the age groups n=25, for the study sites n=30 and for the RDT negative population n=50.
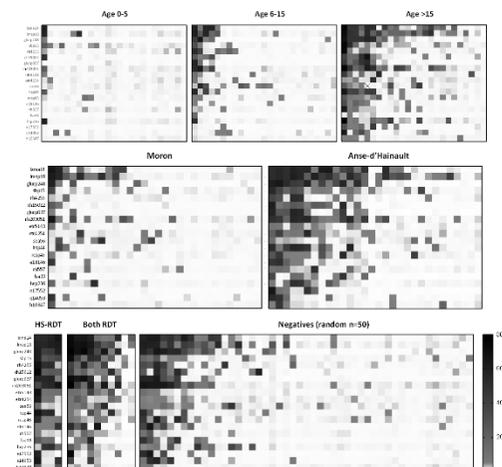

Fig. 3 Heat maps of antimalarial antibody levels by age group, setting and RDT status

Conclusions

- Multi-antigenic targets appear to detect patterns in malaria exposure associated with age, transmission intensity and RDT status.

- Combining positivity to multiple antigenic targets shows a higher accuracy compared to AMA1 and/or MSP1$_{19}$ in detecting a current infection by RDT.

- Future work will expand analyses to larger sample sizes in other areas in Haiti to determine malaria transmission.

References

1. The feasibility of malaria elimination on the island of Hispaniola, with a focus on Haiti. CHAI, 2012.
2. www.malariazeroalliance.org
3. Multiple comparisons analysis of serological data from an area of low *P. falciparum* transmission. Rogier et al., Malaria Journal, 2015.

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The use of antimalarial antibody measurements to support elimination activities in Haiti

L.L. van den Hoogen[1], E. Rogier[2], T. Druetz[3], G. Stresman[1], R. Ashton[3], T. P. Eisele[3], A. Existe[4], J. Boncy[4], M. Chang[2], J.F. Lemoine[5], K. Tetteh[1] & C. Drakeley[1] for the **Malaria Zero Consortium**

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Introduction

- Haiti is considered suitable for elimination with low *Plasmodium falciparum* prevalence, parasites sensitive to chloroquine, a relatively inefficient vector and low risk of importation.[1,2]

- Measuring and monitoring malaria is challenging in elimination settings. Antimalarial antibody measurements are a unique metric as they reflect historical and recent exposure to malaria.

- We performed exploratory analyses of multi-antigenic IgG responses in relation to malaria exposure in central Haiti.

Methods

1. Easy-access-groups (EAG) survey

- Filter-paper blood spots from health facilities, schools and churches were collected in two communes in central Haiti: Verrettes and La Chapelle (Fig 1)[1]. Participants were tested with highly-sensitive (HS-) RDTs and conventional RDTs.

- We screened IgG responses to 23 *P. falciparum* antigens using a bead-based assay.[3] Median fluorescence intensity was log-transformed and standardised by antigen using z-scores.

2. Test positivity rates (TPR)

- Test positivity rates (RDT or microscopy positive/total population tested at the health facility) were collected between January and August 2017 for seven health facilities which were also included in the EAG study (Table 1).

Table 1 Population size in seven health facilities included in the EAG study and for which TPR was collected.

	EAG study	TPR
Total population tested at health facilities	1756	4878
Medium population tested per health facility (IQR)	259 (208 – 290)	748 (546 – 835)

Malaria Risk, Haiti

Predicted Test Positivity Rate (%)
- No to very low risk (<5%)
- Very Low risk (5–10%)
- Low risk (10–15%)
- Low-Medium risk (15–20%)
- Medium risk (20–25%)
- High-Medium risk (25–30%)
- High risk (30–35%)
- Very high risk (>35%)

Verrettes
La Chapelle
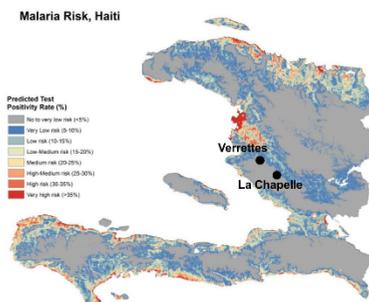
Fig. 1 Predicted malaria risk[1] in Haiti and the two study sites in central Haiti.

Results

- A total of 5998 samples were tested by RDT and the IgG detection assay. Forty-five individuals were positive by RDT (Table 2).

Table 2 General characteristics of the study population, central Haiti

	La Chapelle	Verrettes
N	1944	4054
Median age (IQR)	12 (8 – 23)	14 (8 – 33)
Female	55.8% (1074/1926)	62.5% (2516/4027)
RDT		
- Negative	98.9% (1914/1936)	99.3% (4016/4044)
- HS-RDT only	0.3% (5/1936)	0.0% (0/4044)
- Both RDTs	0.9% (17/1936)	0.7% (28/4044)

- Antibody levels were significantly higher in RDT and HS-RDT positives versus those negative by RDT (p<0.001, Fig 2a).

- Mean antibody levels by health facility showed a positive trend with TPR (Fig. 2c-d). They seem to be more discriminative at lower levels of TPR (<5%) than cross-sectional RDT prevalence (Fig. 2b).
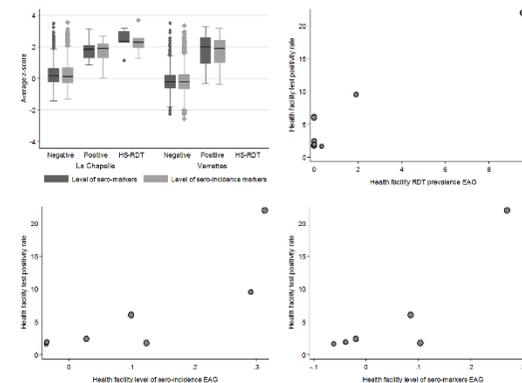

Fig. 2 Averaged z-score across all sero-markers (n=23) and those related to recent exposure (n=6) by RDT status (a). Health facility TPR versus RDT prevalence (b); versus average health facility z-score across all sero-markers (c); and across recent exposure markers (d) during the EAG study. Sizes of dots in plots b-d are adjusted for TPR population size (y-axis).

Conclusions

- Antibody responses from the EAG survey appear to show additional granularity at lower levels of TPR versus cross-sectional RDT prevalence in seven health facilities in Haiti.

- Applying decision rules to antibody levels in a population may help in stratification of malaria control and intervention activities.

- Future work will focus on the selection of antigen combinations that optimally represent malaria transmission intensity in this study area.

References

1. The feasibility of malaria elimination on the island of Hispaniola, with a focus on Haiti. CHAI, 2012.
2. www.malariazeroalliance.org
3. Multiple comparisons analysis of serological data from an area of low *P. falciparum* transmission. Rogier et al., Malaria Journal, 2015.
4. Novel serological biomarkers provide accurate estimates of recent Plasmodium falciparum exposure for individuals and communities. Helb et al., PNAS, 2015.

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Antimalarial antibody detection assays: in search of a standardised tool to confirm the absence of malaria transmission

Lotus L. van den Hoogen[1], Paolo Bareng[2], Julio Rodrigues[3], Gillian Stresman[1], Nuno Sepulveda[1], Kevin K. Tetteh[1], Ralph Reyes[2], Malou Macalinao[2], Tom Hall[1], Katie Patterson[1], Tate Oulton[1], Joana Alves[3], Jennifer Luchavez[2], Fe E. Espino[2], Kimberley Fornace[2], Alan Kitchen[4], Peter Chiodini[5] & Chris Drakeley[1]

[1]London School of Hygiene & Tropical Medicine, London, UK. [2]Research Institute for Tropical Medicine, Department of Health, Muntinlupa City, the Philippines. [3]National Institute of Public Health, Praia, Ilha de Santiago, Cabo Verde. [4]NHS Blood and Transplant, Hertfordshire, UK. [5]Hospital for Tropical Diseases, London, UK.

Background

- Antibody measurements are unique as they reflect historical and recent exposure to malaria. They may be better suited than prevalence of infection in low transmission settings.
- There is no standardised test to detect antimalarial antibodies for epidemiological use. Commercial assays are available to screen donor blood products[1].
- We compared antibody detection assays for their relative performance, with a focus on supporting claims of absence of malaria transmission.

Methods

Specificity
Cross-reactivity
Ease-of-use
(Tab. 1)

Samples from two eliminating settings
(Fig. 1-2 & Tab. 2)
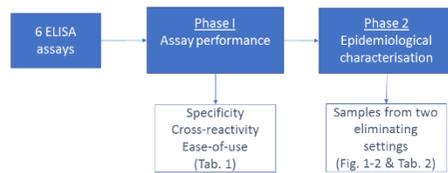
ELISA: Enzyme-linked Immunosorbent Assay
(i.e. antibody detection assay)
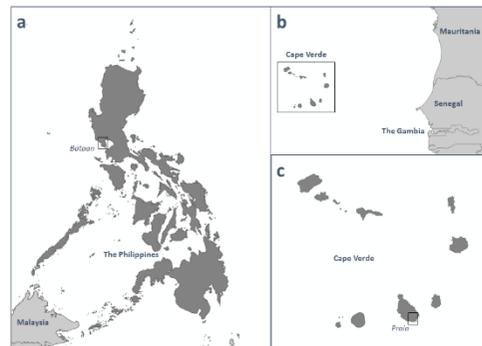
Figure 1 Map of the included study areas and surrounding countries in the Philippines (a) and Cape Verde (b & ca). Map c is zoomed in from the square in map b. Bataan: the last indigenous case reported in 2005. Praia: historically low but unstable transmission since the 90s.[2,3] Samples were collected prior to the outbreak in 2017.

Results

Phase I: Assay performance

Three out of six assays were taken forward for epidemiological evaluation (Table 1). Assay B was no longer available at the start of Phase II.

Table 1 Sample volume needed, specificity, cross-reactivity and ease-of-use for all assays.

	A	B	C	D	E	Research based
Amount of serum	Medium (50 μL)	Medium (50 μL)	High (150 μL)	Low (2 μL)	Low (10 μL)	Low (2 μL)
Specificity	High 99%	High 99%	High 98%	Low 82%	High 98%	High 98%
Cross-reactivity	Low 0%	Low 1%	Low 0%	High 16%	Low 2%	Low 3%
Ease-of-use	High	High	Low	Medium	Medium	Medium

Specificity: proportion correctly identified as negative using 223 malaria non-exposed samples. Cross-reactivity: proportion incorrectly identified as positive using 191 toxoplasma-infected, malaria non-exposed samples. Ease-of-use: e.g. need for sample preparation and whether reagents are ready-to-use.

### Phase II: Epidemiological characterisation
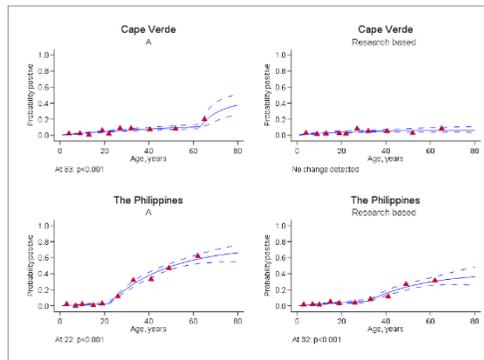
Figure 2 Seroconversion plots for the Philippines & Cape Verde. A simple reversible catalytic model was fitted to seroprevalence by age deciles (blue fit with 95%CI). Seroprevalence is shown per age decile in red triangles. The timing of statistically significant change points and associated p-values are noted below the plots.

Table 2 Results for two selected assays in the Philippines and Cape Verde.

	The Philippines (n=1994)		Cape Verde (n=1429)	
	A	Research based	A	Research based
Seroprevalence	19%	9%	7%	4%
1/5	1% (3/235)	1% (3/235)	3% (3/116)	4% (4/116)
6/15	1% (5/479)	1% (6/479)	1% (4/319)	2% (5/319)
>15	30% (342/1132)	13% (152/1132)	9% (84/961)	5% (47/961)
Proportion seroconverts/year (recent)	0.1%	0.2%	0.3%	0.3%
Predicted EIR[4]	<0.01	<0.01	<0.01	<0.01
True seroprevalence	0% in ≤20 years old	0% in ≤20 years old	0% in ≤20 years old	0% in ≤20 years old

There was no recent malaria transmission detected in both settings by two of the assays taken forward (Figure 2 & Table 2). Assay E was not fit for epidemiological characterisation due to inconsistent age prevalence patterns and general prevalence estimates.

Discussion

- Two assays correctly identified the absence of exposure to malaria in the youngest age groups in both settings.
- Small differences in estimates were seen between these two assays. This may be due to a higher serum concentration and greater blocking capacity in commercial assay A.
- Future work will aim to optimise the research-based assay further for epidemiological use in eliminating settings.

References

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2. UCSF & MAP. Country briefing: Eliminating malaria in Cape Verde. Accessed online at globalhealthsciences.ucsf.edu/pub, 2015.
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Combining serological and clinical incidence metrics from easy–access group surveys and routine health surveillance to guide elimination activities in Haiti

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Background

- Haiti is considered suitable for elimination with low *Plasmodium falciparum* prevalence, parasites sensitive to chloroquine and low risk of importation.[1,2]
- However, heterogeneity is pronounced and there is limited insight as to how cases detected through routine health surveillance (RHS) relate to malaria transmission in the community.
- We aimed to 1) identify which antigens induced antibody responses related to historical and recent exposure, and 2) compare serological measures from easy-access groups (health centers and schools) to routine health surveillance data to support Haiti's elimination activities.

Methods
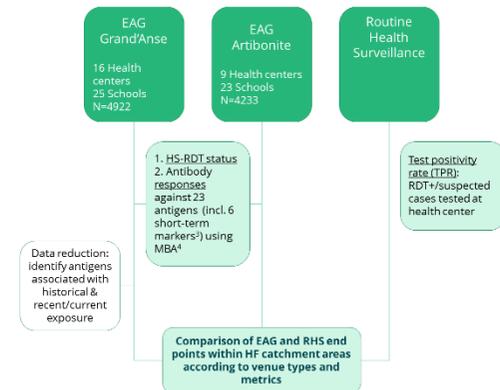
Figure 1: Flow-chart of methodology outlining data sources and analyses. EAG: Easy-access group survey. HS-RDT: highly sensitive rapid diagnostic test. MBA: Multiplex bead assay. RHS: routine health surveillance.

Location of study areas in Artibonite and Grand'Anse are shown in Fig. 3A.

Results

- Antibody responses were classified as markers of historical exposure if they showed moderate correlation with age (Pearson's rho >0.35). For the remaining targets, random Forest analysis was used to classify antigens as recent markers if they were strong predictors of HS-RDT positivity (mean decrease in accuracy >15).
- Antibody responses against historical (n=4) and recent (n=2) markers were combined and classified as positive using the mean +2 SD of the lower distribution using finite mixture models.
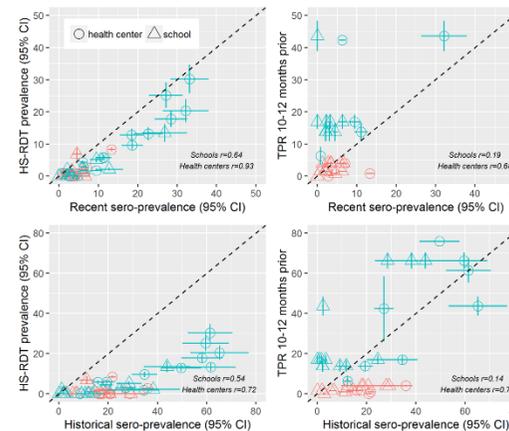

Figure 2: Comparison of prevalence estimates by venue type in Grand'Anse (blue) and Artibonite (red). TPR: test positivity rate (for schools the nearest health center was used); CI: confidence interval.

- Generally, sero-prevalence for recent markers and populations tested at health centers showed a stronger correlation with HS-RDT prevalence and TPR (Fig. 2).
- Health center catchment areas were predicted using geographical data (e.g. altitude, roads) and EAG data (e.g. households for a subset of participants were geolocated).
- Arbitrary thresholds of sero-prevalence against recent markers in health centers were used to map intervention profiles according to predicted health center catchment areas in Grand'Anse (Fig. 3).
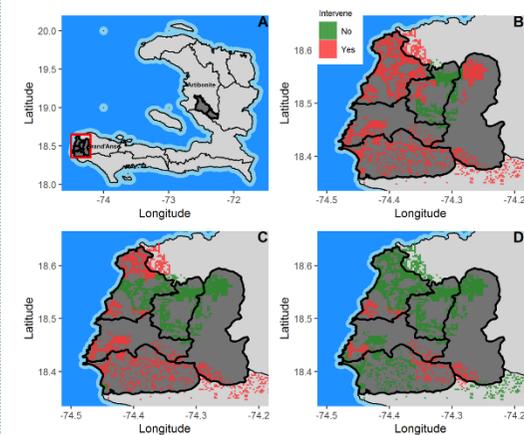

Figure 3: Maps showing study areas in Haiti in dark gray with Grand'Anse highlighted by the red box (a). Areas requiring intervention in Grand'Anse using thresholds of (b) 10%, (c) 20% and (d) 30% recent sero-prevalence at local health centers. Light gray areas are neighbouring areas while green and red areas are predicted health center catchment areas.

Discussion

- This approach allowed for the identification of spatial clusters for targeting interventions.
- Future work will include investigation of seroconversion rates and age stratification as well as the use of single antigenic markers.
- Serological metrics from EAG surveys may help in identifying communities with ongoing malaria transmission to guide elimination activities in Haiti.

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4. Rogier et al. Multiple comparisons analysis of serological data from an area of low *Plasmodium falciparum* transmission. Malaria Journal, 2015.

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