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Malaria Elimination in Ethiopia:
Relevance of Molecular/Diagnostic Tools in Epidemiological Studies

Ashenafi Assefa Bahita

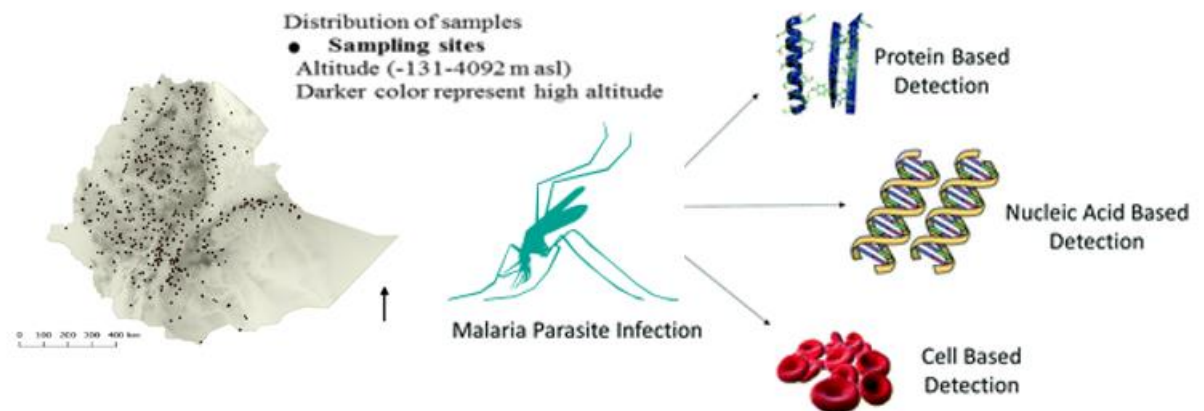
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Ashenafi Assefa Bahita



Dissertation for the Degree of Doctor of Philosophy (Ph.D.) in Public Health, School of Public Health, College of Health Sciences, Addis Ababa University



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Malaria Elimination in Ethiopia: Relevance of advanced Molecular/Diagnostic Tools in
Epidemiological Studies

A Dissertation Submitted to the School of Graduate Studies, Addis Ababa University, in Partial
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This dissertation is dedicated to my late father,

Assefa Bahita G/michael

(1940-August, 2017)

List of original papers:

This thesis is based on the ensuing original research articles

1. **Paper I:** Glucose-6-phosphate dehydrogenase (G6PD) deficiency in Ethiopia: absence of common African and Mediterranean allelic variants in a nationwide study. Malaria Journal, volume 17, 2018. DOI: 10.1186/s12936-018-2538-4
2. **Paper II:** Multiplex serology demonstrate cumulative prevalence and spatial distribution of malaria in Ethiopia. Malar Journal volume 18, 2019. DOI: 2019 10.1186/s12936-019-2874-z
3. **Paper III:** Evaluation of subpatent malaria infection in North Western Ethiopia. Malar Journal volume 19, 202. DOI: 10.1186/s12936-020-03177-w

Acronyms and Abbreviations

AAU	Addis Ababa University
AAUSPH	Addis Ababa University School of Public Health
ACT	Artemisinin-based Combination Therapy
AMA-1	Apical Membrane Antigen-1
aOR	Adjusted Odds Ratio
ASTMH	American Society of Tropical Medicine and Hygiene
BCC	Behavioral Change Communication
BF	Blood Films
BG	Benishangul-Gumuz
BSA	Bovine Serum Albumin
CDC	Center for Disease Control and Prevention
CI	Confidence Interval
CSA	Central Statistical Agency
DBS	Dried Blood Spot
DEFT	Design effect
DHS	Demographic Health Survey
DIHA	Drug Induced Hemolytic Anemia
DNA	Deoxyribonucleic Acid
EA	Enumeration Area
EIR	Entomological Inoculation Rate
ELISA	Enzyme Linked Immunosorbent Assay
EMIS	Ethiopia Malaria Indicator Survey
EPHI	Ethiopian Public Health Institute
FDA	Food and Drug Administration
FMM	Finite Mixture Model
G6PD	Glucose-6-Phosphate Dehydrogenase

G6PDd	Glucose-6-Phosphate Dehydrogenase deficiency
GFATM	Global Fund to Fight AIDS, Tuberculosis and Malaria
GIS	Geographic Information System
GLURP	Glutamate-Rich Protein
GMEP	Global Malaria Eradication Program
GSH/GSSG	Glutathione reduced (GSH) Glutathion oxidized (GSSG)
GST	Glutathione-S-Transferase
GTP	Growth and Transformation Plan
GTS	Global Technical Strategy
HH	Household
HMIS	Health Management Information System
HMP	Hexose Monophosphate
HRP2	Histidine Rich Protein 2
HSDP	Health Sector Development Program
HSTP	Health System Transformation Plan
IgG	Immune globulin G
IRS	Indoor Residual Spray
ITN	Insecticide Treated Nets
LAMP	Loop-mediated isothermal amplification
LLIN	Long-lasting insecticidal nets
LM	Light Microscopy
LoD	Limit of Detection
LSHTM	London School of Hygiene and Tropical Medicine
Masl	Meters above sea level
MDG	Millennium Development Goal
MERG	Monitoring and Evaluation Reference Group
MFI	Mean Fluorescence Intensity
MICS	Multi Indicator Cluster Surveys

MIS	Malaria Indicator Survey
MoH	Ministry of Health
MSP	Merozoite Surface Protein
MSP-1	Merozoite Surface Protein-1
MTI	Malaria Transmission Intensity
NADP+	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Reduced form of Nicotinamide Adenine Dinucleotide Phosphate
NERRC	National Ethics and Research Review Committee
OD	Optical Density
OR	Odds Ratio
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDA	Personal Digital Assistance
Pf	<i>Plasmodium falciparum</i>
PHEM	Public Health Emergency Management
pLDH	pan-Plasmodium lactate dehydrogenase
Pm	<i>Plasmodium malariae</i>
PMI	President Malaria Initiative
Po	<i>Plasmodium ovale</i>
PPP	Pentose Phosphate pathway
PPS	Probability Proportional to Size
PQ	Primaquine
PR	Parasite Rate
Pv	<i>Plasmodium vivax</i>
RBM	Roll Back Malaria
RDT	Rapid Diagnostic Test
RFLP	Restricted Fragment Length Polymorphism
RNA	Ribonucleic Acid

RT	Room Temperature
SCR	Seroconversion Rate
SNNPR	Southern Nations and Nationalities People's Region
SPR	Slide Positivity Rate
SRS	Simple Random Sampling
SUFI	Scaling Up for Impact
TARE	Telomere Associated Repetitive Element
TFQ	Tafenoquine
UN	United Nations
WHO	World Health Organization
WMR	World Malaria Report
WRAIR	Walter Reed Army Institute of Research

Operational Definitions

- **Asymptomatic malaria:** refers to a positive malaria test by microscopy, RDT, or Polymerase chain reaction (PCR) in the absence of any clinical signs or symptoms.
- **Enumeration area (EA):** refers a unit of land delineated for the purpose of enumerating housing units and population; used during national population census.
- **G6PD deficiency:** refers a genetic disorder in which a person doesn't have enough of an enzyme called glucose-6-phosphate dehydrogenase (G6PD) that helps red blood cells work normally. This was measured by PCR-RFLP method.
- **Low Transmission setting:** refers to a setting where Slide Positivity Rate (SPR) in febrile cases <5%, prevalence 0-10% and Entomological Inoculation Rate (EIR) <0.25.
- **Malaria control:** refers reducing the disease burden to a level which is no longer a public health problem.
- **Malaria diagnosis, parasitological:** refers diagnosis of malaria by detection of malaria parasites or Plasmodium-specific antigens, antibodies or genes in the blood of an infected individual, using microscopy, RDT, PCR or serology methods.
- **Malaria elimination:** refers interrupting local mosquito-borne malaria transmission in a defined geographical area, i.e. zero incidence of locally contracted cases.
- **Malaria eradication:** refers the permanent reduction to zero of the worldwide incidence of malaria infection caused by a specific agent; i.e. applies to a particular malaria parasite species.
- **Malaria seroprevalence:** refers a measure of malaria exposure prevalence by using the antimalarial antibodies in a blood sample, show previous exposure to malaria infection. Individual samples were considered positive, if they had a positive antibody response either for Merozoite Surface Protein-1 (MSP-1) or apical membrane Antigen-1 (AMA-1) antigens for either *P. falciparum* or *P.vivax*. Individual samples were considered positive for *P.malariae* or *P.ovale*, if they had a positive antibody response for species specific Merozoite Surface Protein-1 (MSP-1) antigens either *P. malariae* or *P.ovale*.
- **Molecular biology** refers to the study of biology on a molecular level including the structure, function, and makeup of biologically important molecules such as DNA, RNA, and proteins.

- **Molecular epidemiology** refers to the study of the contribution of potential genetic and environmental risk factors, identified at the molecular level to the etiology, distribution and prevention of disease within and across populations.
- **Subpatent malaria infections** refers to infections below the detection threshold of microscopy or rapid diagnostic tests (RDTs), but detectable with Polymerase Chain Reaction (PCR).

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Executive Summary

Background:

Malaria is still among the major diseases of public health importance in Ethiopia. Ethiopia presents a diversified ecological situation, resulting in a highly variable eco-epidemiology of malaria. Following the scale-up of antimalarial interventions in the past two decades, malaria burden has significantly declined leading to the National Malaria Control Program (NMCP) in Ethiopia to re-embark on a strategy for step by step nationwide malaria elimination. Learning from the failed malaria elimination endeavors of the 1960s, achieving such an ambitious target, given the complicated eco-epidemiology of malaria in Ethiopia may require several inputs and evidences.

Measures of malaria burden and transmission dynamics using conventional diagnostic methods [microscopy and Rapid Diagnostic tests (RDTs)] may be incomplete, particularly, in low and seasonal transmission settings, where few infections are detected. Unlike most parts of Africa, *P. falciparum* and *P. vivax* co-exist in Ethiopia. Malaria elimination requires determining the actual burden, distribution as well as detection and cleaning of all forms of malaria infection. The knowledge gap in the occurrence, prevalence and distribution of Glucose-6-Phosphate Dehydrogenase (G6PD) deficiency has been a limiting factor for radical cure of relapsing malaria and transmission interruption. The group of drugs within 8-aminoquinolines, such as Primaquine and Tafenaquine, are the only available treatment of relapsing malaria. Eight-aminoquinolines can induce severe hemolysis in G6PD deficient individuals. The study used advanced tools to investigate the epidemiological risk factors relevant for malaria elimination.

Objective:

This study used serology and molecular methods to describe the malaria (*Plasmodium spp*) burden and distribution, as well as to determine G6PD deficiency prevalence and allelic types, in order to produce reliable evidence for malaria elimination in Ethiopia.

Methodology:

Dried blood spot (DBS) samples collected in 2011 and 2015 as part of the national household Malaria Indicator Surveys (MIS) were used. The Ethiopian Malaria Indicator Surveys (EMISs)

utilized a multi-stage cross sectional surveys representing the various malaria epidemiological settings in Ethiopia. EMIS-2015 samples were investigated using bead-based multiplex assays for IgG antibodies for six *Plasmodium spp* antigens: four human malaria species-specific merozoite surface protein 1 19kD antigens (MSP-1) and apical membrane antigen 1 (AMA-1) for *P. falciparum* and *P. vivax*. Seroprevalence was estimated by age group, elevation, and administrative regions. Seroconversion rates were estimated using a reversible catalytic model fitted with maximum likelihood method. Sub samples of EMIS-2015 from three administrative regions (Amhara, Tigray and Benishangul Gumuz regions) were screened by Microscopy, RDTs and nested Polymerase Chain Reaction (nPCR) for malaria parasites and results were compared to determine prevalence of subpatent infections. A randomly selected subset of samples from EMIS-2011 were genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique and three common G6PD genotype variants: G6PD*A (A376G), G6PD*A- (G202A) and Mediterranean (C563T) were investigated.

Data were analysed using Stata 13 (College Station, USA). Serology data were generated from a multiplex instrument as Mean Fluorescent Intensity minus background (MFI-bg). To dichotomize seropositivity, log-transformed MFI-bg values were fitted to a two-component Finite Mixture Model (FMM) by the FMM procedure with normal distribution and maximum likelihood estimation outputs. A seropositivity cutoff value was determined by the mean MFI-bg value of the first (assumed seronegative) component plus three standard deviations. *Plasmodium falciparum* and *P. vivax* seropositivity were defined as being positive for either or both MSP-1 and AMA-1 antigens. Species specific MSP-1 antigens were used for *P. malariae* and *P. ovale* seropositivity.

EMISs sampling weights were used to ensure the representativeness of the samples tested to the study population. Adjustments were made by region, elevation, and age group. Linear and multiple logistic regression models with 95% confidence intervals (CI) were employed to determine the association of risk factors with *Plasmodium spp* infection. Differences in distributions were evaluated using Chi square (χ^2) test with P value < 0.05 considered significant. Spatial analysis and geographical mapping were done using QGIS and ArcGIS softwares.

Main Findings:

National seroprevalence for antibodies to *P. falciparum* was 32.1% (95% CI: 29.8-34.4) and 25.0% (95% CI: 22.7-27.3) for *P. vivax*. Estimated seroprevalence for *P. malariae* and *P. ovale* were 8.6% (95% CI: 7.6-9.7) and 3.1% (95% CI: 2.5-3.8), respectively. Seroprevalence estimates were significantly higher at lower elevations (<2000 m) compared to higher elevations (2000-2500 m), for *P. falciparum* [Adjusted Odds Ratio (aOR) 4.4 (95% CI: 2.7 -7.0), $p < 0.01$].], although evidence was weak for *P. vivax* [aOR 1.5 (95% CI: 0.9-2.3), $p > 0.05$]]. Among administrative regions, *P. falciparum* seroprevalence ranged from 11.0% (95% CI: 8.8-13.7) in the Somali to 65.0% (95% CI: 58.0-71.4) in Gambela Region. Seroprevalence for *P. vivax* ranged from 4.0% (95% CI: 2.6-6.2) in the Somali to 36.7% (95% CI: 30.0-44.1) in Amhara Region. Models fitted to measure seroconversion rates showed variation nationally and by elevation, region, antigen type and within species.

Malaria DNA screening using nPCR from three regions (Amhara, Tigray and Benishangul Gumuz) detected 3.3% (95% CI: 2.7-4.1) positive cases. *P. falciparum* accounted for 3.1% (95% CI: .5-3.8), *P. vivax* 0.4% (95% CI: 0.2-0.7), mixed (*P. falciparum* and *P. vivax*) 0.1% (95% CI: 0.0-0.4), mixed (*P. falciparum* and *P. malariae*) 0.1% (95% CI: 0.0-0.3). nPCR detected nearly three-fold more positives compared to microscopy. Sixty one percent of the nPCR positive cases were from Benishangul Gumuz Region.

The G6PD genotyping study showed the more severe G6PD deficiency allelic types, G202A (A-) and C563T (Mediterranean), to be absent in the current study. A national prevalence of 8.1% G6PD*A (A376G) mutation variants was observed with regional variation, with highest prevalence observed in Tigray Region (13.7%) to none in Harari. Of the G6PD*A (A376G), 31% were hemizygous males and 62.1% and 6.8% were heterozygous and homozygous females, respectively.

Conclusion and Recommendations:

The current study used multiplex serology and serological markers to report the malaria exposure burden and transmission intensity of the four human malaria species. The study documented heterogeneity in malaria burden and transmission over different elevations, administrative regions,

and age groups. Malaria exposure was by far higher compared to the active infection reported by microscopy and RDTs. *P. falciparum* sero prevalence increases with decreasing elevation, whereas *P. vivax* slightly increases with elevation in the study setting up to 2,500 m; showing *P. vivax* is more prevalent in highlands compared to *P. falciparum*. Variation was observed on the spatial distribution and dynamics of transmission over the regions. The northwestern part of the country is carrying the largest burden of malaria compared to the east. Among the regions, Gambela and Benishangul-Gumuz had the highest burden of malaria transmission. The current study documented the presence of *P. malariae* and *P. ovale* in all administrative regions. Given that *P. ovale* possesses a hypnozoite stage, its control and elimination requires programmatic attention. The seroprevalence results may be used as baseline data for the future malaria elimination efforts and may help the NMPCP in tailoring intervention approaches.

The current study documented a considerable proportion of subpatent *Plasmodium* spp infections undetected by microscopy. Such subpatent infections are potentially infective to mosquitoes, contributing for malaria transmission in addition to their debilitating chronic effect on the individuals affected. Efficient malaria elimination efforts have to address the impact of subpatent infection on transmission and health.

In this study, the more severe variants G6PD *A- (G202A) and Mediterranean (C563T) mutations were not observed. The G6PD *A (A376G) mutation observed is a mild variant resulting in close to normal (85%) enzyme activity of a non-deficient person, without significant clinical manifestations of G6PD deficiency related hemolysis. This study investigated three of the most important and potentially expected mutation types in the study area among the hundreds of known G6PD variants worldwide. Although the study cannot definitively conclude the absence of any clinically important G6PD deficiency, it suggests a low risk of hemolysis, and confirms the utility of the recently adopted Primaquine treatment without prior G6PD testing. The risks and benefits of Primaquine radical cure without G6PD testing may need to be further assessed in Ethiopia as the *P. vivax* and *P. ovale* case management is evolving and may incorporate higher dose and shorter course regimens of Primaquine and Tafenoquine.

In summary, the current work used advanced serological and molecular diagnostic tools to produce evidence to the epidemiological factors that may be relevant for malaria elimination. It also emphasized the need for assessing and introducing advanced diagnostic techniques, such as PCR and multiplex serology to provide releable evidences required towards malaria elimination in Ethiopia.

Key words: Malaria Elimination, G6PD deficiency, Asymptomatic Infection, Subpatent infection, Seroprevalence, Multiplex serology, Microscopy, RDT, nPCR, Ethiopia

1. INTRODUCTION

1.1. Global malaria burden

Malaria is a very old disease which probably originated from Africa and accompanied human migration to the Mediterranean shores, India and south east Asia (1). In the past, malaria used to be common in the marshy areas around Rome, where its name mal-aria meaning, bad air given to the disease which was also known as Roman fever (1,2). Malaria is caused by the Protozoa of the genus *Plasmodium* and transmitted by the bite of female anopheles mosquitoes. It is primarily disease of the tropics and sub tropics. It is widespread in the humid regions of Africa, Asia and south and Central America. Five parasite species are known to cause malaria in human beings; *Plasmodium falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*, a zoonotic species of known to infect humans (2).

The intricate interaction between host, parasite and vector are the major factors in the epidemiology and complexity of the parasite. The epidemiology of malaria can be viewed in terms of being stable (stably endemic) or unstable (unstably endemic, similar to epidemic prone areas). Stable malaria refer to a situation in which there is a measurable incidence of natural transmission for six or more months, over several years (2,3). Areas that experience only seasonal transmission on the other hand, are referred to us unstably endemic areas (3,4). People who live in stably endemic (hollo endemic) areas usually develop high levels of immunity and tolerate the malaria infection better with few or no symptoms at all.

Transmission of malaria is affected by geographic physiognomy of a country and often coincides with the rainy season. The geographic physiognomy of a country that refers to the latitude position, the altitudinal variation, including the faces of the uplands, adjacencies to water bodies, forests, and desserts, etc(4). Epidemic malaria is observed in areas of low to moderate endemicity or to areas previously free of malaria among non-immune people. These outbreaks can usually be attributed to change in human behavior or change of the environment (5). Climate change, global warming and other climatic events play a role in increasing the risk of malaria transmission in

previously malaria-free areas, presumably because the associated weather conditions encourage vector breeding and parasite sporogony (2,6).

Humans are the only significant reservoirs for the malaria parasite and sustained transmission depends on contact between infected humans and anopheline mosquitoes and vice versa (2,7). The malaria transmission capacity of mosquitoes depends not only on its ability to support sporogony, but also on factors such as feeding habits, longevity and density (8). Climate and ecological factors can affect mosquitoes' behavior and population (9). Pools of infected humans serve as reservoirs of the parasite; favoring the survival of parasites breeding close to human habitations (9,10). The risk for severe disease is generally confined to children less than five years of age and immunocompromised persons including pregnant women (11). The highest disease risk was observed among populations exposed to low to moderate intensities of transmission in paradox with high transmission intensity areas (11).

The recent world malaria report estimated that 219 million cases of malaria and 435, 000 malaria deaths occurred in 2017 worldwide (12). 92% of the cases and 93% of all malaria deaths occur in Africa. South East Asia and South America carry the remaining burden of malaria (12). The majority of malaria deaths occur among children under five years of age, especially in remote rural areas with poor access to health services (11,13). Close to all cases of malaria are caused by *P. falciparum* and *P. vivax*. In 2017; 99.7% of malaria cases in Africa, 62.8% in South East Asia, 69% in Mediterranean Region and 71.9% in the WHO Pacific Region were caused by *P. falciparum*. *P. vivax* attributed to 74.1% of the malaria cases in the American Region (12).

Malaria disproportionately affects the poor and the poorest of the world populations (14,15). There has been an extensive debate on the relationship between malaria and poverty. We know intuitively that poor people living in a low socio-economic environment, unable to access prevention and control methods are more likely to become infected than people living in better housing, perhaps can afford air-conditioning, access to appropriate diagnosis and treatment. Malaria affects productivity and work absenteeism ultimately contributing to the “poverty trap” in which so many people in rural Africa find themselves stuck. In most instances, malaria occurs in harvesting seasons and absence from work for few days has serious impact on productivity. Although the

burden of malaria is high on the poor, some researchers and policymakers argue that malaria is a disease of the tropics and its determinants are mainly ecological and climatic factors (8,14–16).

Nevertheless, today, with the shrinking of the global malaria distribution map the disease burden and distribution is in line with the global socio economic indicators. The least developed countries have the highest burden of malaria and women, young children and people living in less accessible areas are most affected (17). According to the 2018 WHO world malaria report, 15 countries in the sub Saharan Africa and India carried out 80% of the global malaria burden (12). Four countries in sub Saharan Africa, Nigeria, Democratic Republic of the Congo, Mozambique and Uganda contribute about 45% of the global malaria cases (12).

1.2. Global progress in malaria control

Over the past few decades remarkable achievements have been made in malaria control worldwide. Generally the number of malaria cases have declined (10,12,17–19). The World Malaria report 2018 stated that there were 20 million fewer malaria cases in 2017 compared to 2010 (12). Significant gain was recorded in the WHO South East Asia Region where malaria has declined by 59%. However, Africa shows a resurgence in malaria cases in 2017 compared to 2016. Ten of the highest burden countries in Africa have reported increase in the number of malaria cases in 2017 (12). But changes vary by region and countries. Rwanda and Ethiopia were exceptions by reporting decreasing malaria cases on the African Continent. Rwanda reported 430, 000 fewer cases and Ethiopia reported 420, 000 fewer cases in 2017 compared to 2016 (12).

Between 2000 and 2013, 47% of malaria mortality were averted, leading to the prevention of an estimated 4.3 million deaths (20). About 93% of the deaths in 2017 occurred in Africa, however, Africa accounted for 88% of fewer malaria deaths reported in 2017 compared to 2010 (12).

Global investment for malaria control efforts has been more or less stable since 2010 (17). In 2017, an estimated 3.1 billion USD was invested for malaria control and elimination activities; 624 million insecticide treated mosquito nets were distributed between 2015 and 2017 that lead to an estimated 50% of vulnerable population to have access to insecticide treated nets (ITN) and with

40% of people at risk to have at least one ITN for two persons (12). A total of 276 million rapid diagnostic tests (RDTs) were globally distributed in 2017. Between 2010 and 2017 an estimated 2.74 million treatment courses of artemisinin based combination therapy (ACT) were produced and distributed. ACT treatment was targeted to patients who tested positive to malaria. In sub-Saharan Africa, up to 52% of under-five children with fever were diagnosed and treated for malaria (12).

Strong malaria surveillance is critical for identifying cases and deaths due to malaria. Surveillance information help in identifying groups and people at an increased risk for malaria and help targeting resources for maximum impact. In Africa, by 2017, 36 out 42 moderate to high burden countries reported up to 80% of their public health facilities had achieved complete surveillance reporting (12,21).

With declining numbers of malaria cases more sophisticated methods and tools are now required to detect, estimate and predict malaria cases. Advanced and sensitive diagnostic tools, digital surveillance systems, new vector control tools and insecticides, effective drugs for acute treatment and radical cure, mathematical and spatial methods /models have been developed in the last two decades. The substantial gains in malaria control can be in part attributed to these technological advancements, making malaria control investment cost effective: investments in malara control result in one of the highest returns in public health (17).

1.3. Malaria elimination initiative, progresses and challenges

The WHO Global Malaria Eradication Program (GMEP) was launched in 1955 and aimed to eliminate malaria from 143 countries (22–24). It was successful in eliminating malaria from 37 countries but was abandoned in 1969 (20,22,25–27). After GMEP period countries were focusing on the control of malaria, mainly with diagnosis and treatment. During the last two decades, the global approach on malaria control is shifting from control towards elimination. The change was made possible by the presence of new tools such as insecticide treated nets (ITNs), insecticide spraying, improved diagnostics tools (Microscopy and Rapid diagnostic tests (RDTs), Artemisinin based Combination Therapy (ACT) and most importantly the availability of funds (23). In 1998,

the Roll Back Malaria (RBM) was initiated to accelerate malaria control intervention in Africa using the scale up for impact (SUFI) approach (24). Following the success of SUFI many countries in Africa declared their intent to malaria elimination. In 2009, the WHO African Region Office adopted a resolution to accelerate control to achieve malaria elimination (24).

Between 2001 and 2014, a substantial expansion of malaria control interventions contributed to massive reduction of malaria related morbidity and mortality (12). In the same period, one of the global milestones, the Millennium Development Goal six (c) (MDG6c) “*to have halted by 2015 and begun to reverse the incidence of malaria and other major diseases*” was met (17,28) . However, the gains registered are fragile and unevenly distributed. There were some instances where resurgence of malaria was recorded (12). To keep the momentum and maintain the downward trend of malaria morbidity and mortality, the WHO endorsed the Global Technical Strategy for Malaria 2016–2030 (GTS) in 2015 (28). Among the ambitious, but attainable objectives of the GTS are to reduce malaria morbidity and mortality by 90% and to eliminate malaria from at least 35 countries by 2030. The GTS listed milestones for 2020 and 2025 (28). In line with the GTS objectives, the number of countries reporting progress is increasing. Between 2007 and 2019 eight countries eliminated malaria and in 2017, 26 countries reported less than 100 endogenous cases (an indicator that elimination is within reach) increased from 15 countries in 2010 (28). On May 22, 2019 Fedela Chaib (WHO communication officer) announced that Algeria and Argentina were certified to be free of malaria (media communication). Algeria is the second country in Africa after Mauritius to be certified in 46 years. Malaria was first discovered in 1880 in Algeria by the French physician Dr Charles Louis Alphonse Laveran (29).

The malaria control and elimination effort has been confronted with several challenges that slow down the progress and sometimes reverse gains (20,23,30,31). The following are among the major challenges: a) lack of sustained and predictable funding from local and international sources, compounded by security issues and a lack of political commitment and regional collaboration b) the emergence of resistance, both for antimalarial drugs and insecticide resistance by mosquitoes. Both are serious impediments for the effectiveness of malaria control acceleration efforts c) weak performance of health system, surveillance, monitoring and evaluation. This comprises of the inefficiency to track gaps in program coverage and monitor changes in burden and distribution of

the disease among others d) in countries where *P. falciparum* and *P. vivax* co-exist, reducing the burden of *P. vivax* is very difficult, due to undetectable and difficult to treat liver stage parasites (hypnozoite) that result in relapse and thereby contributing to disease transmission. Vivax elimination is further complicated by the high prevalence of Glucose-6-Phosphate Dehydrogenase (G6PD) deficiency. Treating G6PD deficient people with 8-aminoquinolines (the only antimalarial drug class that can clear the hypnozoite) may lead to hemolysis e) many people who are infected with malaria parasites remain asymptomatic or subpatent; they are not visible for the health system. Those people harbor parasite densities too low to be detected by the routine diagnostic methods such as microscopy and RDTs, but still contribute for the transmission of the disease.

Global attempts have been made in terms of increasing political commitment, mobilizing investment and developing new tools that support surveillance, vector control, diagnostic and treatment against malaria. We used the available advanced diagnostic and molecular methods to generate evidence required for the global malaria control effort, in particular evidence required for the malaria control and elimination program in Ethiopia.

1.4. Malaria elimination requirement and new tools

The global malaria eradication is envisaged by several United Nations (UN) and WHO organs (24,28,32). WHO developed the Global Technical Strategy (GTS) document with milestones for step by step global action towards malaria eradication (28). The UN Secretariat Office together with the Bill and Melinda Gates Foundation developed a document entitled “From Aspiration to Action”, which aspires to the global eradication of malaria by 2040 (33). The global political attention following the remarkable gains in malaria control and increasing availability of resources in the past decade encouraged several malaria endemic countries to envisage malaria elimination for their respective countries (12). Several innovative tools are being produced and promise to support the malaria elimination effort; although most are not yet ready to malaria control and elimination activities. On the other hand, some distinguished scientists with rich experience in malaria elimination (such as Professor Gao Qi, a WHO malaria elimination advisor from Jiangsu Institute of Parasitic Diseases, China), advise that intensifying the innovative employment of available control tools may be a short cut towards malaria elimination for low and middle income

countries. For example the 1-3-7 malaria control approach has been instrumental towards malaria elimination in China (34).

Besides the development of new tools that may improve diagnosis, treatment, prevention, surveillance and response towards malaria elimination; the achievements in the past decades were considerable (34). This may include: Long Lasting Insecticide Treated Nets (LLIN), several groups of insecticides which are safe and effective, that may replace pyrethroids; besides the effective and fast acting ACTs, short course Primaquine (seven days) and Tafenoquine (single dose), both 8-aminoquinolines are now available for radical cure of vivax malaria (35). Point of care tools for the diagnosis of G6PD deficiency are entering the global market. Highly sensitive malaria test kits that may be used for the detection of subpatent infection are becoming available (36,37). Robust and sensitive diagnostic methods that use nucleic acids and antigens are available for advanced surveillance and referencing (37–41,41,42). There is no malaria vaccine currently available that can completely protect from malaria, but there is a promising first generation malaria vaccine (RTS, S) that has a protective efficacy of up to 50 percent during the first year following administration (43). The further development, customization and adoption of those tools are expected to enhance and accelerate malaria elimination and its eventual global eradication (37).

1.5. Malaria in Ethiopia

Malaria is still among the major public health challenge in Ethiopia. The disease is recognized by local people named differently, with local vernaculars such as “Nidad” in Amharic “Asso” Tigrigna, “Bussa/Kendo” Afan Oromo. It was formally recognized as a major public health problem in 1950s and then, the Ethiopian Government started control activities as a pilot project. Later, it was further strengthened and Ethiopia was a partner with the earlier discussed WHO global initiative to eradicate malaria in 1959 (44,45).

Malaria transmission in Ethiopia is heterogeneous and seasonal, with peak periods following the main rains from June to September and short rains from March to April (46). Historically, malaria epidemics occurred at five to eight-year intervals. The last major malaria epidemic, in 2004/2005 caused 2 million clinical cases and claimed approximately 3,000 lives (47,48).

Although malaria transmission has been declining over the past decades (49,50), 60% of the Ethiopian landmass is still suitable for malaria transmission and the communities residing in this area remain vulnerable for malaria transmission. Malaria distribution is primarily associated with altitude and rainfall (47,51,52). The National Malaria Control Program (NMCP) of Ethiopia considers areas below 2,000 m elevation as malaria endemic and hence, eligible for malaria control activities (53). *Plasmodium falciparum* and *P. vivax* are the major documented malaria parasite species, with approximately 1.1 million *P. falciparum* cases and 470,892 *P. vivax* cases reported in 2017 (18). The proportion of *P. vivax* cases fluctuates over time. A slight relative increase in *P. vivax* cases has been documented with the decline in *P. falciparum* incidence. The overall proportion of *P. vivax* remained in a range of 30-40% (54–56). Malaria is among the weekly reportable diseases in the Public Health Emergency Management (PHEM) system in Ethiopia (53).

1.5.1. Malaria control and gains

Since 2004/2005, the Federal Ministry of Health (FMoH) developed and employed three strategic plans with five years intervals, the latest extends from 2014 to 2020 (53,57,58). These plans are in line with the Health Sector Development Program (HSDP) of the Ministry of Health (59). In all the strategies, areas lower than 2,000 meters of elevation (<2,000 m) are considered ‘malaria-endemic’ and are targeted to receive key malaria control interventions including long-lasting insecticidal nets (LLINs), indoor residual spraying (IRS), prompt diagnosis, using microscopic examinations of blood smears and/or rapid diagnostic tests (RDTs), prompt case management with ACT or Chloroquine (for *P. vivax* mono-infections) and behavioural change communication (2,10,11-13).

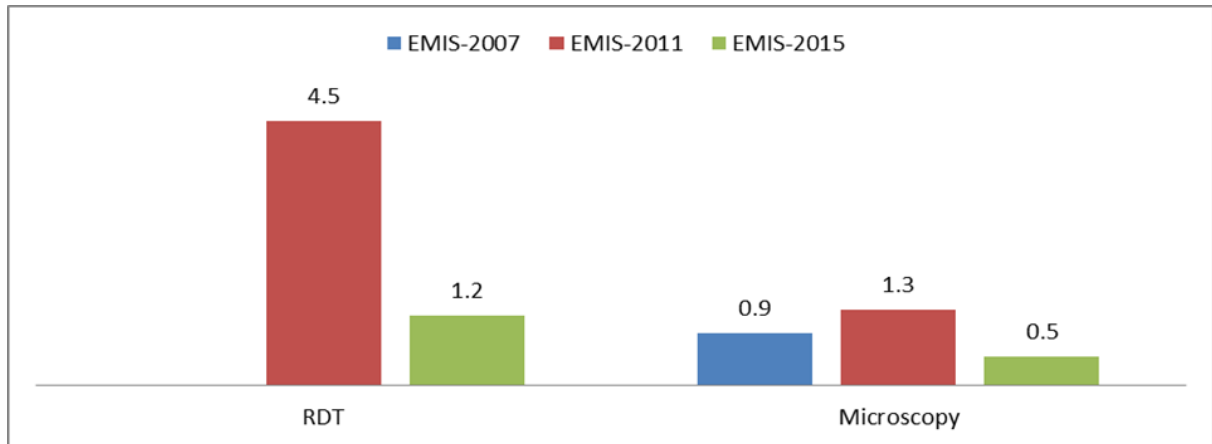


Figure 1 National malaria prevalence using microscopy and Rapid Diagnostic Tests (RDTs), Ethiopia Malaria Indicator Survey 2007, 2011 and 2015 (55,60,61).

Several reports documented a declining trend in malaria burden in Ethiopia (49,50,53,56). Although malaria indicator surveys have the limitation of being a point survey the last three Ethiopian Malaria Indicator Surveys (EMIS) reported very low prevalences using microscopy and RDTs (Figure 1): 0.9% by microscopy only in 2007, 1.3% by microscopy and 4.5% by RDTs in 2011, and 0.5% by microscopy and 1.2% by RDTs in 2015. This change was in line with the increasing accessibility to malaria control and prevention methods over the period (55,60,62). A hospital based time series analysis between 2001 and 2011 showed that malaria morbidity and mortality has significantly decreased in 2011 compared to the base line (2001 - 2005). Confirmed malaria cases declined by 66% and slide positivity rate declined by 37% in 2011 compared to the period between 2001 -2005. Malaria inpatient cases were 54% lower and malaria deaths were 68% lower in 2011 compared to the period 2001 and 2005 (49). The decline in morbidity and mortality was again in line with intervention scale up. The number of malaria cases and deaths remains very high in under five children 81% and 73%, respectively.

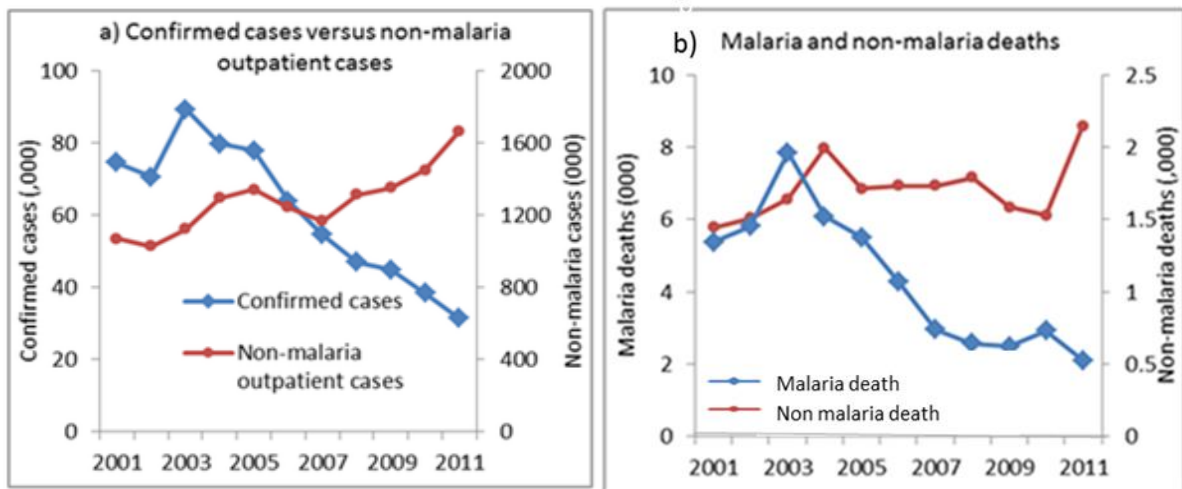


Figure 2: Declining trend of malaria between 2001 and 2011 from hospital based data in Ethiopia (49). a) Confirmed cases versus non malaria outpatient cases b) Malaria and non-malaria deaths.

Several studies documented the declining trend of malaria in the nation. A study by Hiwot et al (56) showed that malaria incidence rate dropped significantly from an average of 43.1 cases per 1000 population annually between 2001 and 2010 to 29.0 cases per 1000 population annually between 2011 and 2016. Accordingly, the study documented that malaria mortality decreased from 2.1 deaths per 100 000 people annually between 2001 and 2010 to 1.1 deaths per 100 000 people annually between 2011 and 2016. The study further documented a significant decrease in the areas with malaria transmission. By 2016 high transmission was confined to the western parts of the country adjacent to the remote international border areas. Between 1990 and 2015 a dramatic 94.8% decline in mortality was reported (50)

The gains in malaria control were guided by a strong NMCP at the FMOH (53,55,57,59,63). The NMCP works around three major areas in line with the WHO and RBM recommendations: 1) vector control, 2) early diagnosis and treatment and 3) prevention and control of epidemics (24,64). These strategies are supported by massive development of human resources, increase in malaria knowledge and practice, monitoring and evaluation and operational research (63). The human resource development in Ethiopia is particularly successful in terms of Health Extension Program (HEP), where the number of Health Extension Workers (HEW) have reached to more than 40, 000 scattered all over the country by the day (53,65). The NMCP has distributed more than 60 million

insecticide treated mosquito nets between 2006 and 2016 (56). Diagnosis of malaria cases by RDT or microscopy increased from less than one million before 2008 to more than eight million in 2014 (53). ACTS and Chloroquine are readily available in health centers. The proportion of facilities with ACTs in-stock remained >87% during 2006–2011(49,53).

1.5.2. Malaria elimination in Ethiopia

Ethiopia was among the priority countries for the WHO GMEP during the late 1950s. Although malaria was eliminated in 37 countries of the world, mainly in Europe and America further progress stalled and the WHO shifted from an eradication strategy to control in several countries including Ethiopia in the early 1970s (25–27). In Ethiopia, the complex and heterogeneous malaria risk distribution and transmission pattern as well as the low socio-economic status of the nation contributed to the failure. There are only limited references regarding the processes, achievements and challenges during the eradication period in Ethiopia. Potentially similar experiences were however documented by Madagascar and Swaziland (26). During field work, the student observed utensils, labels and even old cars scattered around malaria control offices and compounds all over the country, which were used during the malaria eradication program in Ethiopia. A concerted effort would be required to organize and document the lessons from the malaria eradication program in 1970s before the last eye witnesses have died. These may be critical for the evolution of the current malaria elimination undertaking.

Ethiopia has sustained its coverage of interventions and demonstrated progressive reduction in malaria burden and death since 2004/2005 (49,50,56). It was one of the first countries to embrace the Scaling Up for Impact (SUFi) concept for malaria control (24,45,66). The declining trend observed in malaria burden in line with the sustained coverage in malaria interventions led to the development of the fourth ambitious national strategic plan (53,58). The recent strategic plan, include malaria elimination objectives among others (51). Amid the goals of the National Strategic Plan for Malaria Prevention, Control, and Elimination 2014–2020 were to achieve the following by 2020 (51):

- Near zero malaria deaths (no more than 1 confirmed malaria death per 100,000 population at risk)

- Reduce malaria cases by 75% from baseline of 2013
- Eliminate malaria in selected low transmission areas

The current gains in malaria prevention and control match the WHO requirements for a country to move from malaria control to pre-elimination or elimination (53,64,65). The global malaria community is advocating malaria elimination and eventual eradication. Although the concepts and practices of malaria elimination are not new to Ethiopia, the re-introduction of malaria elimination plans could bring the country into a cross road, similar situation as the late 1950s GMEP did. The lessons learned in the 1950s and 1960s should ensure that Ethiopia does not repeat similar failures as the GMEP.

Since May 2019, malaria case have built up and outbreaks have been reported in a number of woredas/districts in six regions (Amhara, Oromia, SNNP, Tigray and Beneshangual Gumuz regions). These observations are in line with similar reports from neighboring countries such as Burundi, Uganda and Sudan. NMCP and PHEM are organizing efforts to react and contain the situation (personal communication NMCP and PHEM officers).

2. STATEMENT OF THE PROBLEM

Malaria elimination is a complex endeavor which requires a tailored approach with appropriate tools for detecting, treating and preventing rare and resurgent malaria cases (34,37,67). The recent national strategic plan demands evidence and tools for a better understanding of the malaria situation in Ethiopia in order to guide the development of informed policies and strategies towards malaria elimination (53). In this thesis, the transmission dynamics, and approaches to radical cure and interruption of transmission are addressed.

- 1) Due to restrictions in infrastructure and limitations of the study methods, the true prevalence and intensity of malaria transmission is not clearly defined in Ethiopia. The current reporting system, including the routine Health Management Information System (HMIS), and PHEM are incomplete (53). The three national malaria indicator surveys (EMIS 2007/2011/2015) were point surveys and may be sub-optimal for reporting of an acute disease like malaria. New methods may help to address this knowledge gap.
- 2) With the declining malaria prevalence, low density parasitaemia that cannot be detected by conventional methods such as RDT and microscopy play an important role in transmission dynamics and challenges malaria elimination effort (68). The magnitude of the asymptomatic, subpatent parasite reservoir can now be estimated using advanced molecular methods (68).
- 3) Malaria elimination requires the interruption of transmission for all malaria species in a given area (28,65). As *P. falciparum* and *P. vivax* co-exist in Ethiopia, it is mandatory to use appropriate therapy to clear *P. vivax* hypnozoite stage. There is only one class of drugs available for the radical cure of *P. vivax* infection: the 8-aminoquinolines. Primaquine and Tafenoquine (30,69–73). The safe use of 8-aminoquinolines requires a solid knowledge on the distribution of G6PD deficiency in a population. G6PD is an enzymopathy that can result in haemolysis when a deficient person is exposed to a range of drugs including 8-aminoquinolines as risk factors. Thus, detecting the presence of G6PD deficiency and determining its epidemiology and spatial distribution are critical for malaria elimination.

- 4) Although there is evidence for the decline of malaria transmission in Ethiopia (49,56), the transmission pattern as well as the geospatial transmission and clustering of the disease have not been investigated and documented at a national level.

3. RATIONALE OF THE STUDY

Global malaria eradication was attempted in the 1950's. WHO launched the Global Malaria Eradication Program (GMEP) in 1955 in a number of countries endemic for malaria (27). Thirty seven countries succeeded in eliminating malaria, whereas many others, including Ethiopia went back to control strategies in the early 1970s (44). Currently, the global as well as the national malaria burden is declining (12,49,50,53,56). Building on this progress, the NMCP in Ethiopia has re-embarked on an ambitious strategy to step by step eliminate malaria starting from low transmission settings (53,65). Learning from the experience of the 1960s, such an endeavor needs to be supported by strong research evidences. Assessing vectorial capacity, parasite epidemiology supported by more sensitive diagnostic tools and strong surveillance needs to be emphasized.

The MISs reported malaria prevalence to be 1.3% in 2011 and <1% in 2015 using microscopy, and 4.5% and 1.2% using RDT, respectively. With such a low malaria prevalence, transmission can be expected to be focal in some areas. Advanced tools, such as parasite DNA detection by Polymerase Chain Reaction (PCR) and advanced serological methods may help to delineate malaria burden /prevalence and determine focal areas of transmission. Once the focal nature of malaria transmission is confirmed, an effort will be undertaken to eliminate parasites from those targetted area.

An efficient drug regimen that clears the parasite from the patient body is critical for focal transmission managment. Several optionsof Artemisinin based Combination Therapy (ACTs) are available for the successful management and control of *P. falciparum*. However, the 8-aminoquinoline is the only group of drugs that can produce radical cure of *P. vivax* and *P.ovale* [Primaquine (PQ) and Tafenoquine (TFQ)]. TFQ has passed the last phase of clinical trial and is approved by the American Food and Drug Administration (FDA), but yet to be approved by the WHO (69,74).

The WHO recommends combining ACT with a single dose PQ for the interruption of *P. falciparum* (including mature gametocytes) (75). A 14 day PQ regimen is required to completely cure *P. vivax* by killing the hypnozoites and preventing relapses (radical cure). A recent multi

country clinical study has proved that a short course PQ regimen (7 days) is not inferior to a PQ 14 day regimen. The short course is expected to alleviate the adherence problems associated with the 14 day PQ regimen (76). Worldwide, more than 17 countries mainly in Southeast Asia have included the radical cure with PQ as first line treatment for *P. vivax* malaria in their national policy (77). In Ethiopia, where *P. falciparum* and *P. vivax* co-exist malaria elimination has to target both species. Due to safety concerns, G6PD testing is recommended before PQ is used for the radical cure. However, G6PD testing followed by the radical cure will have a significant effect on feasibility and cost effectiveness as well as the achievable population coverage of large scale programmatic interventions. Large scale studies are required to determine the prevalence and distribution of G6PD deficiency and to guide evidence based decision making for the NMCP in Ethiopia.

This study addresses the above national programmatic needs by utilizing advanced molecular and epidemiological methods. Efforts were made to determine the sero-epidemiology of malaria parasites, prevalence of subpatent infections and the prevalence and spatial distribution of G6PD deficiency using nationally representative samples.

4. LITERATURE REVIEW

(N.B: A separate systematic review was conducted for malaria serology, asymptomatic infections and G6PD deficiency. A summary of the review is presented below)

Since the establishment of the Roll Back Malaria (RBM) by WHO 1998, the funding modalities for malaria control have increased. The advent of financing mechanisms, such as the Global Fund to fight HIV, TB and Malaria, the US President Malaria Initiative (PMI) and Bill and Melinda Gates Foundation have provided funds towards this goal (24). The reduction of malaria related morbidity and mortality, using intensive control measures through the availability of resources followed by malaria elimination and eventual eradication is increasingly becoming the consensus approach recommended by the WHO and the global malaria community. A number of endemic countries are aspiring to achieve malaria elimination and become certified to be free of malaria (29).

There is an overlapping use of the terms malaria eradication, elimination and control. According to WHO the three terms are defined as follows (25):

- Malaria **control** refers to reducing the disease burden to a level at which it is no longer a public health problem.
- Malaria **elimination** refers to interrupting local mosquito-borne malaria transmission in a defined geographical area, i.e. zero incidence of locally contracted cases.
- Malaria **eradication** is the permanent reduction to zero of the worldwide incidence of malaria infection caused by a specific agent; i.e. applies to a particular malaria parasite species.

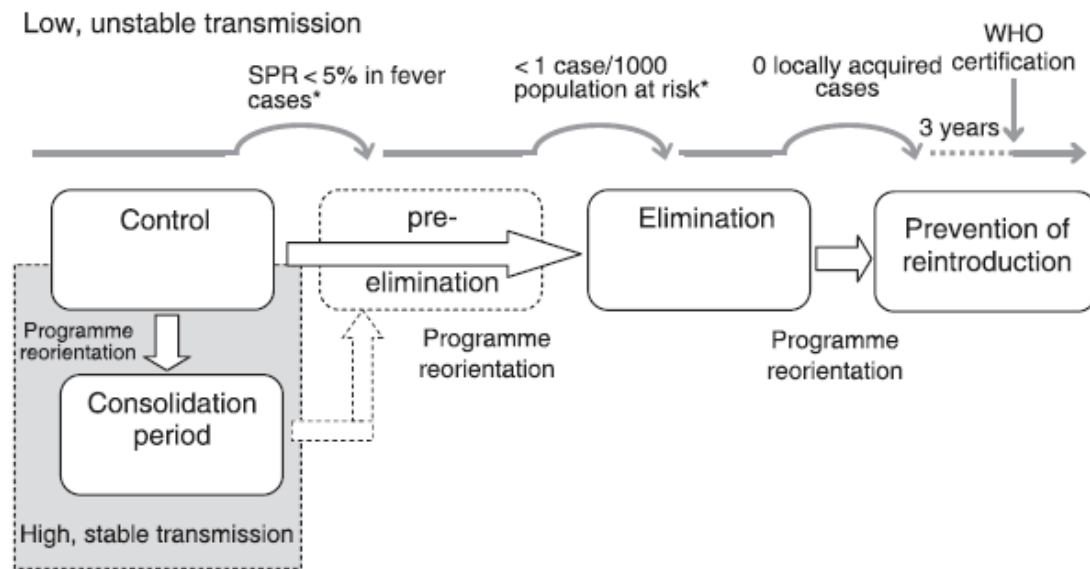


Figure 3. The malaria control and elimination continuum in high and low transmission settings (77) (* those milestones are indicative only; SPR = slide positivity rate).

With the strategic move from malaria control to elimination (figure 3), the requirement of advanced tools for active surveillance for the detection of cases, their complete treatment and containment is essential (25,27,78).

4.1. Molecular diagnostics

Molecular epidemiology focuses on the contribution of potential genetic and environmental risk factors, identified at the molecular level to the etiology, distribution and prevention of disease within and across populations, adding another dimension by providing information that classical epidemiology cannot provide. It's not an emerging field, as most epidemiological investigations are now supported by molecular techniques (37).

The current study utilized molecular methods to address major epidemiological questions related to malaria: burden and transmission, prevalence of subpatent infections and G6PD deficiency in Ethiopia.

4.1.1. Malaria serology

Malaria transmission intensity is an important determinant for malaria burden. The severity of the disease and mortality rate increases with increasing transmission intensity, especially in children less than five years of age (13,54,79–81). Mapping malaria transmission across the nation is a prerequisite for planning and implementing control and elimination strategies. The monitoring of transmission over time and assessing the impact of intervention activities is essential in order to respond to changing transmission levels in time (51,58,73,82).

In areas where transmission intensity is low (Slide Positivity Rate (SPR) in febrile cases <5%, 0–10% prevalence and Entomological Inoculation Rate (EIR) <0.25) and malaria control programmes are considering switching from sustained control to elimination (figure 3). Monitoring of malaria transmission intensity (MTI), and targeting interventions to foci experiencing higher malaria transmission will be vital (83–85). Sustained control efforts backed by strong monitoring and surveillance may lead to the successful elimination of malaria (64,73). However, in areas where transmission has been reduced substantially, new challenges arise for monitoring and evaluation. Estimates of transmission derived from health facility records through routine methods tend to be unreliable. They often either over or under estimate health care utilization (79,84–88). In addition passive surveillance misses large numbers of subpatent/asymptomatic infections (68,79,85,89–91). This may include, patients who don't seek healthcare for other reasons and in some instances patients attending the private health care system.

Measurements of malaria transmission can inform interventions, but current measures have significant drawbacks. The number of parasites detected in human blood is influenced by several factors; such as acquired immunity, drug use and drug resistance, seasonal variation in transmission (2). A more complete understanding would require frequent sampling, which is challenging. With patient parasitaemia too low and short lived to be detected, periodic parasite prevalence surveys may still miss or under estimate areas of low and sporadic transmission. In addition low parasite density infections can be below the detection level of conventional methods (microscopy/RDTs) and require high-tech tools and highly skilled staff for detection (68,80,84,85).

In low transmission settings, traditional measures of Malaria Transmission Intensity (MTI) such as the entomological inoculation rate (EIR) and parasite rates (PR) lack sensitivity because

numbers of positive samples (mosquito and human) are low. EIR, the mean number of infectious mosquito bites per individual per year, requires catching and dissecting mosquitoes with high skill to establish the percentage of infected mosquitoes. This measurement becomes increasingly challenging as endemicity decreases. It also lacks precision since mosquito distributions are markedly heterogeneous (85,87,92,93). Further, both entomological and parasitological measures are affected by seasonality (6).

The ideal tool for assessing malaria endemicity would measure what percentage of the population is infected and for how long. Serology could address some of these requirements. In contrast to the lifespan of the vector or the half-life of individual infections, exposed individuals can remain seropositive for antimalarial antibodies for extended periods of time (84,85,94,95), though which antibody (e.g., MSP1-19, AMA1) persists for how long remains debatable (12). Antibodies persist markedly longer than individual malaria infections or infected mosquitoes, which suggests that seroprevalence rates could provide a proxy measurement for malaria endemicity (80,85,94–96). Serological markers of transmission show greater utility in low transmission areas, as low seroprevalence reflects little to no cumulative exposure.

Therefore, sero-epidemiological studies could serve as important tools for the assessment of malaria transmission and changes in burden following the implementation of successful control programmes. While it has become easier to measure antibodies, there remain considerable challenges to analyzing and interpreting antibody levels (97).

Although several serological studies have been conducted in low transmission settings in Africa (Tanzania, Zambia, Gambia) and South East Asia demonstrating the usefulness of the method in prevalence studies and malaria transmission pattern studies (80,84,85,88,93,97). A Study in Somalia uses serologic methods to describe variability of infection in low transmission setting (83). In the Gambia serological study samples from the last two decades showed the precise decline of malaria in line with other malaria metrics (98). However, a study in Uganda indicated the limitations of serologic methods in a high transmission setting that required a model for adjustment and better estimation of transmission (99). Serology studies are very rare in Ethiopia. Few studies conducted in Oromia, Amhara and Southern Nations, Nationalities and Peoples (SNNP) regions of Ethiopia reported high seroprevalence compared to microscopy and RDTs (54,100–102). Asheton et. al conducted serological studies in Oromia Region from samples collected in a school based cross sectional survey (54,100). The results reported a wide variation of prevalence between

microscopy and serology for *P. falciparum* (0–50% versus 0–12.7%) and *P. vivax* (0–53.7% versus 0–4.5%) for serology and microscopy respectively. A study by Yalew et al., reported a 30.0% and 21.8% seroprevalence for *P. falciparum* and *P. vivax*, respectively, for the Amhara Region in 2013 (102). The studies recommended use of serological methods for large scale studies, such as the Ethiopian Malaria Indicator Surveys (EMISs). In this thesis an effort was made to use samples collected during the EMIS 2015 to report a nationwide malaria seroprevalence estimate.

4.1.2. Asymptomatic/ Subpatent infection

In countries approaching elimination, diagnosis and surveillance shift from controlling morbidity and mortality to detecting infections and measuring transmission. In malaria elimination, the clearing of all forms of the parasite from a given community is required (25). In many malaria endemic countries, two or more species of malaria co-exist. In Ethiopia *P. falciparum* and *P. vivax* co-exist in approximately 3:1 ratio (51,53,55,57,58,60). (Little or no emphasis has been given on the occurrence and distribution of the other rare species.

In endemic settings malaria can be easily recognized presumptively and with standard diagnostic methods, such as microscopy and RDTs. However, increasing evidence is emerging regarding the high prevalence of asymptomatic infections that cannot be detected by the standard diagnostic techniques (100,101). Asymptomatic *Plasmodium* spp infections are defined by a positive test by microscopy, RDT, or Polymerase chain reaction (PCR) in the absence of any clinical signs or symptoms (68,84,90,91,93,105,106). However, they are important in transmission dynamics of malaria. Understanding, quantifying and establishing their dynamics in transmission may be critical in malaria control and elimination.

In the malaria control phase, standard microscopy and RDT are enough for detecting and treating malaria morbidity and mortality. However, parasites are difficult to detect when densities are low. With declining parasite density, more sensitive tools, such as PCR are required for the detection of parasites and determination of parasite species.

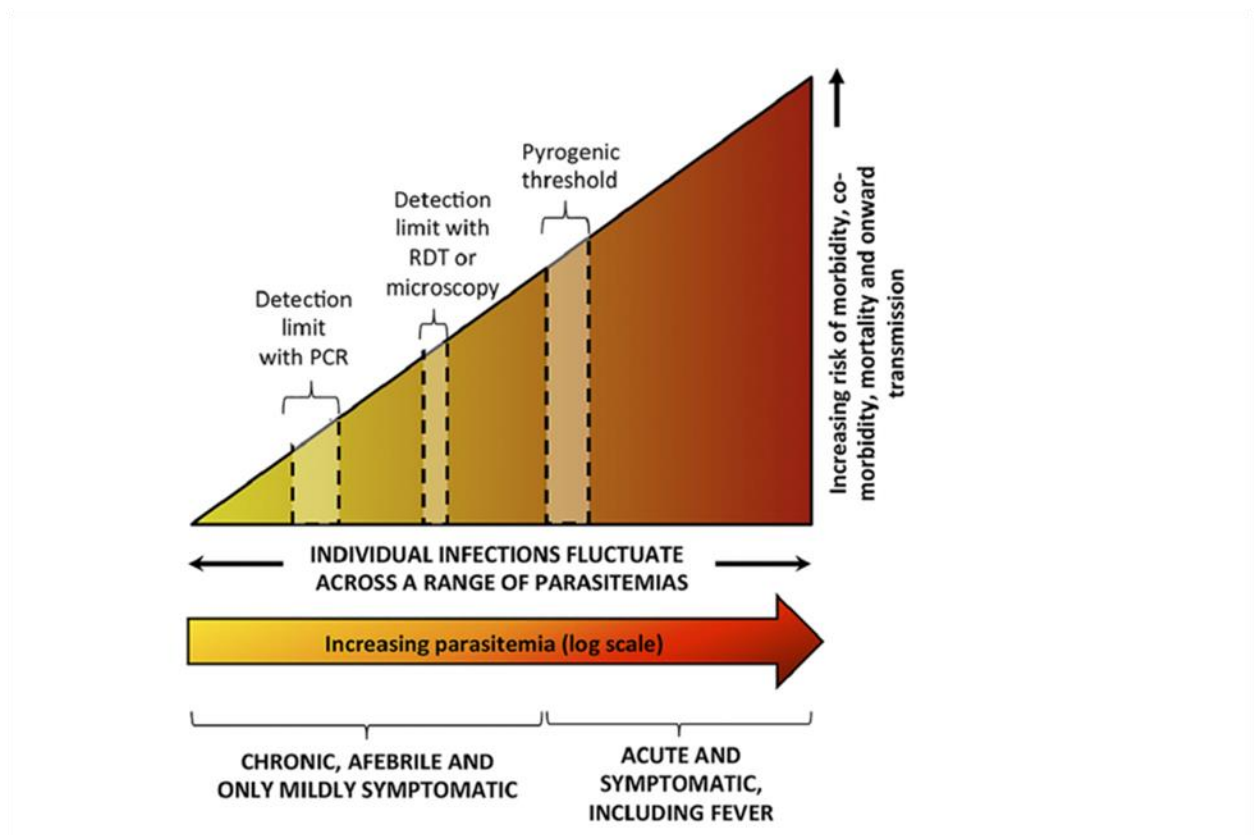


Figure 4. Spectrum of malaria infection. The figure shows increasing risks of morbidity, co-morbidity, mortality, and onward transmission as density of parasitemia increases. [Adapted from (106)].

Theoretically, asymptomatic infections are expected in higher transmission settings due to acquired immunity. Hence asymptomatic infection in low transmission settings may be more difficult to explain. However, high prevalence of asymptomatic malaria are being reported in low transmission settings. It is currently accepted that patent symptomatic infections are only the ‘tip of the iceberg’, while asymptomatic/subpatent infections may represent a large proportion of the population in endemic communities, Figure 5 (107–109).

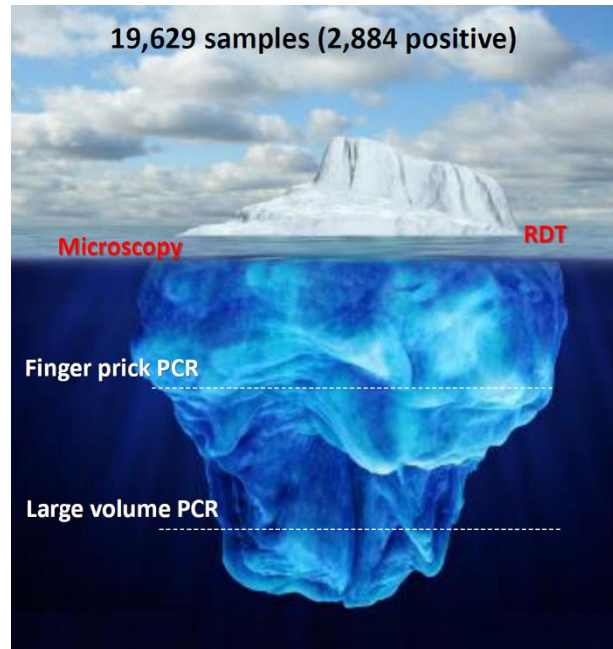


Figure 5. Symbolic representation of asymptomatic infection densities as an iceberg and the detection limits of the various methods (107-109). (Off 19,629 positive samples, only 2884 detected by conventional methods (Unpublished, Jimée Hwang, personal communication).

4.1.2.1. Asymptomatic infection prevalence

A literature survey was made on Pubmed using the phrase “asymptomatic malaria infection in low transmission settings”. The summary of the results is discussed below. The literature survey showed that most reports on asymptomatic infections focusing on low transmission setting were reported or published since 2014 (67%), suggesting a recent increase in knowledge on asymptomatic infection in low transmission settings. Most of the research findings came from Asia, Latin America and Pacific Islands, countries currently progressing towards malaria elimination (18).

Among the current studies reviewed 22/24 reported the occurrence of asymptomatic infections in low transmission settings. The detection of parasites (in particular low density) depends on the sensitivity of the tool used (Figure 5). The prevalence of asymptomatic infections range between 0.003 to 44%. Some of the major findings are summarized below:

A study in the Solomon Islands reported a PCR prevalence of 8.7%, 3.3 fold higher than that was detected by microscopy (110). In Sri Lanka, a house hold survey showed 31% of cases detected were subclinical (111). A study in Laos used a highly sensitive PCR called ultra-sensitive PCR (uPCR) (112) detected more cases compared to RDT. In addition, the study reported a highly heterogeneous distribution of infections often separated by only short distances. In Iran, 565 asymptomatic patients were screened by LM, RDT and qualitative real time PCR (qRT-PCR) to give 3.0, 2.7 and 19.1 % prevalence, respectively (113). However, the qPCR detected only 20 of the 108 qRT-PCR-positive samples, showing variation in sensitivity of tools. Similar study in Colombia reported that out of 3,029 patients diagnosed, 0.2 % were positive by microscopy and 3.7% were positive by qPCR (114). In Kenya, 23% prevalence of asymptomatic infections with focal clustering in the highlands was reported (115). The prevalence increased during transmission seasons compared to the dry seasons. In a non- endemic setting of South Africa, a 2-3.5% prevalence of asymptomatic *Plasmodium* spp infections were reported (116). The study was conducted in refugee camp in the Kwazulu Natal Region using RDT.

Studies on asymptomatic infection in Ethiopia have been very rare. A study conducted by Tadesse et al (117) reported, in south western Ethiopia, where no cases of symptomatic malaria were reported by microscopy and RDT, nested PCR (nPCR), revealed the presence of 5.25% (29/555) and 4.55% (24/555) *Pf* and *Pv* prevalence respectively. One case of co-infection with *P.falciparum* and *P.vivax* was observed in one of the sites that was also missed by both microscopy and RDT.

A high prevalence of asymptomatic infections, 0.5-2.5 % was reported in pregnant women in Bangladesh (118). The prevalence of asymptomatic infection was 5.4 times higher compared to non-pregnant women and men. Individuals living in study clusters of high symptomatic *P. falciparum* incidence had the highest prevalence of asymptomatic infection showing the transmission potential of asymptomatic infection (118).

Recent PCR studies in Iran (107) and Sri Lanka (111) have reported the absence of any form of parasites, suggesting the effective elimination of malaria in study areas. In Iran, 1000 individuals were evaluated in a community and zero prevalence was reported (107). An earlier study in the same area reported a 1% seroprevalence, affirming that the area was malaria endemic in the recent past. Serological methods are capable of indicating a past infection by detecting antibodies in blood samples (80,84). In Sri Lanka, 1,905 individuals were tested in four areas and zero cases were

reported both by microscopy and PCR (111). Sri Lanka was certified to be malaria free by WHO in 2017 (12).

4.1.2.2. Asymptomatic/subpatent infection infectivity to mosquitoes

The infectivity of very low and non-recognized infections to mosquitoes is important in transmission dynamics (68). Studies conducted in Ethiopia, Gambia, Peru and Thailand, showed infectivity of asymptomatic/subpatent infections to mosquitoes (68,108,119,120). From an experimental study, Tadesse et al reported symptomatic, asymptomatic microscopy-detected, and PCR-detected infections were responsible for 8.0%, 76.2%, and 15.8% of the infectious reservoir for *P. vivax*, respectively (68).

4.1.2.3. Methods on asymptomatic infection diagnosis

The gold standard for malaria diagnosis is Light Microscopy (LM). Microscopy and RDTs have been effectively used for the diagnosis of symptomatic malaria in control settings. Microscopy has a detection limit between 5-100 parasites per microliter based on the skill of the microscopists and the time allocated to read a slide (121). New and sensitive tools detect very low density parasites as low as 0.002 par/ μ L of blood (119). With diminishing parasite density in low transmission settings more sensitive tools are needed to detect and manage the remaining infections (67). The summary table below lists the malaria diagnosis methods and their relative sensitivity in detection of parasites (Table 1).

Table 1. List of published assays and limit of detection (LOD) for *Plasmodium falciparum*, adapted and modified from (119).

Method	Detect/Base	Quantification	Parasites/ μ L Blood
Microscopy	Parasite	Yes/No	5-100
RDT	Antigen	No	10-100
Nested PCR	DNA	No	0.1–10
PCR	DNA	No	0.5
qPCR	DNA	Yes	0.02–3
PCR-based	DNA	Yes/No	0.5–1
LAMP	DNA	No	1–10
RPA	DNA	No	4
qRT-PCR	RNA	Yes	0.002–0.02
(QT-)NASBA	RNA	Yes/No	0.02
TARE-2	DNA	Yes	0.03-0.12
varATS	DNA	Yes	0.06-0.15

(qRT-PCR) quantitative reverse transcription PCR, (NASBA) nucleic acid sequence-based amplification, (LAMP) loop-mediated isothermal amplification, (RPA) Recombinase polymerase amplification , (TARE-2)The telomere associated repetitive element 2, (varATS) var gene acidic terminal sequence

In summary, detection and treatment of the massive asymptomatic/subpatent infection, which is a source of transmission is a requirement for rapid malaria elimination. New and sensitive tools can detect very low parasites ($<1/\mu$ L) in low transmission settings. Mosquitoes feeding on individuals with asymptomatic/sublatent *Plasmodium spp* infections could be infectious, thus, contribute to malaria transmission dynamics. These low density infections may result in resurgence of malaria and even malaria epidemics, when conditions are favorable and control measures are minimum. Therefore, stringent efforts may be required to determine the prevalence of asymptomatic infections and their treatment during malaria elimination endeavors.

4.1.3. Glucose-6-Phosphate Dehydrogenase (G6PD) deficiency and distribution

Malaria elimination requires, a drug that is safe and capable of killing all stages and types of malaria (70,122). The only available drugs that are capable of clearing all stages of malaria parasites are the 8-aminoquinoline group. Primaquine (PQ) and Tafenoquine (TFQ) are the only currently available drugs in the class (69,72–74). The 8-aminoquinolones (in particular Primaquine) has been in use for the last 80 years (47,70). As stated above, this drugs may causes mild to severe hemolysis in group of people who have the hereditary deficiency for an enzyme called Glucose-6-phosphate dehydrogenase (G6PD). The status of the deficiency need to be determined before exposing individuals for this group of drugs. The widespread prevalence of G6PD deficiency across populations in malaria endemic areas have hindered the use of Primaquine, despite its uniquely useful range of therapeutic properties.

4.1.3.1. G6PD deficiency/pathophysiology

Glucose-6-phosphate dehydrogenase (G6PD) enzyme activity is necessary for Red Blood Cell (RBC) survival (Figure 6). G6PD enzyme catalyzes the only metabolic pathway capable of generating reducing power to RBCs lacking mitochondria (120–123). G6PD is an enzyme in the Pentose Phosphate Pathway (PPP) (also known as the hexose monophosphate (HMP) shunt). G6PD converts glucose-6-phosphate into 6-phosphogluconate and is the rate limiting enzyme of this metabolic pathway. It supplies reducing energy to cells by maintaining the level of reduced form of the co-enzyme Nicotinamide Adenine Dinucleotide Phosphate (NADPH). The NADPH in turn maintains the supply of reduced glutathione in the cells. The reduced glutathione monomers (GSH) represent the primary defense against hydrogen peroxide, organic peroxides, and free radicals. When G6PD functions normally, the drain of electrons from the NADPH pool caused by oxidative challenge within the cell prompts the PPP to accelerate according to need, i.e. maintaining an NADP–NADPH equilibrium that strongly favors NADPH. This in turn maintains the oxidized reduced glutathione (GSSG–2GSH) equilibrium strongly in the direction of the reduced state (70,125,127) (Figure 6).

However, in cells that have a mutant and defective G6PD gene, the PPP may, depending upon the extent of the enzyme activity, function at near maximum rate even at steady state redox equilibrium. When an oxidative challenge occurs and the equilibrium of NADP to NADPH shifts to the oxidized direction, the PPP is intrinsically unable to accelerate rapidly enough to force the equilibrium in favor of NADPH. This effectively helps the flow of electrons to GSH, and the equilibrium shifts in favor of GSSG. The oxidants consuming these reducing equivalents, in turn, overwhelm the ability of the cell to provide and damage may then occur. Visible evidence of such occurs in the form of Heinz bodies in the RBC membrane that attend acute Primaquine induced hemolytic anemia (70,125) (Figure 6). Heinz bodies cause the membrane to become rigid, and thus decrease the cells' lifespan.

The G6PD / NADPH pathway is the only source of reduced glutathione in RBC (erythrocytes). The role of red cells as oxygen carriers puts them at substantial risk of damage from oxidizing free radicals, except for the protective effect of G6PD/NADPH/glutathione (70,123,126). People with G6PD deficiency are therefore, at risk of hemolytic anemia in states of oxidative stress. Oxidative stress can result from infection and from chemical exposure to medication and certain foods, such as fava beans, which contain high levels of vicine, divicine, convicine and isouramil, all of which are oxidants (70,123,126).

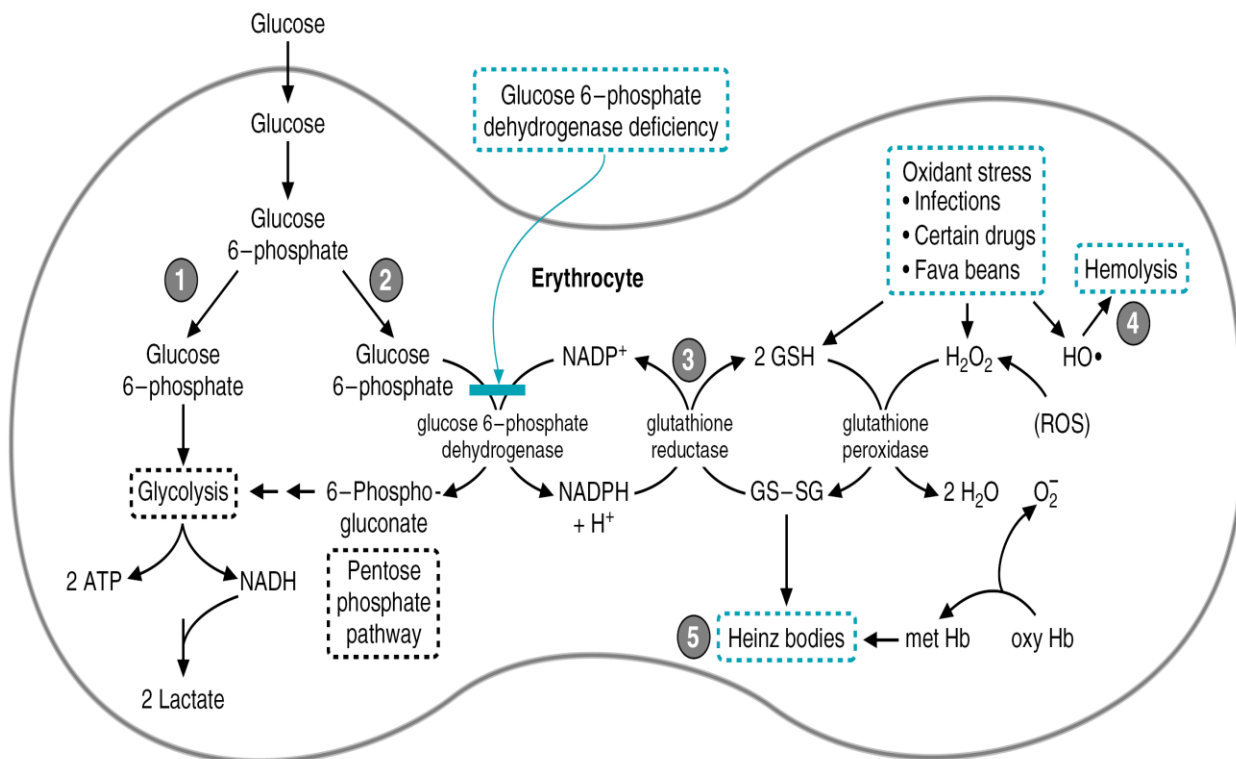


Figure 6 Metabolic pathway for G6PD induced haemolysis. Numbers indicate the metabolic pathway towards toxicity of the cell and hemolysis due to G6PD deficiency (Adapted from presentation ASTMH, 2016)

4.1.3.2. Historical perspectives G6PD deficient

Historically, the most common known trigger of hemolysis has been fava beans (*Vicia faba*), and favism remains, a public health problem in areas where these are common food items and G6PD deficiency is prevalent (125,126,128). Fava beans are unique among other beans since it contain high concentrations of two glycosides, vicine and divicine; and their respective aglycones, convicine and isouramil, are powerful triggers of oxidative stress that cause characteristic hemolytic attacks (70,125,129).

Large majority of G6PD deficient subjects have no clinical manifestations and the condition remains asymptomatic until they are exposed to a hemolytic trigger. However, a hemolysing trigger of the modern day public health significance is the antimalarial Primaquine (and its homologs), a key drug for malaria control as the only licensed treatment against the relapsing liver

stages of *P. vivax* and the asexual blood stages of all species of *Plasmodium spp.* Since its introduction, Primaquine has emerged as a major trigger for hemolysis in G6PD deficient individuals (70,123,125,129).

The uncertainty of a potential hemolysis after Primaquine treatment in G6PD deficient populations in malaria endemic areas has been a barrier to the use of this drug (70,125). Today, in the context of malaria elimination, Primaquine and Tafenoquine are essential tools for which we have no replacement (78).

4.1.3.3. Global distribution of G6PD deficiency

It has been estimated that more than 400 million people worldwide are affected by G6PD deficiency (130). G6PD deficiency affords an evolutionary survival advantage in malaria-endemic regions, as G6PD deficiency confers a degree of protection against severe malaria (123,126,131). The selective advantage of G6PD deficiency under malaria pressure has left its mark in the human genome as a “selective sweep” surrounding the G6PD gene (128). G6PD deficiency is most prevalent in regions where malaria is or was prevalent (Figurer 7). The prevalence of G6PD deficiency is lowest in the Americas, and highest in sub-Saharan Africa (128). The region with the single highest predicted prevalence was in the eastern province of Saudi Arabia (128). The prevalence of G6PD deficiency tended to be lower, but heterogeneous across central and southeast Asia, with “hotspots” identified in eastern India, at the Thai-Lao border and in the Solomon Islands (70). Despite several studies, there remains uncertainty regarding the geographic distribution of G6PD deficiency prevalence in many areas of the world. Models are used to predict global distribution (128). Uncertainty metrics accompanying the modeled map allow prioritization of areas where more community surveys are needed. The prevalence of the G6PD deficiency is far from homogeneous, with considerable variations even within countries, further emphasizing the need for additional data. One limitation of the currently available information is the variability in G6PD deficiency tests used. It is difficult to extrapolate from the reported prevalence of G6PD deficiency the risk of severe primaquine-induced hemolysis (82,125,132). Supplementing the prevalence maps with data on genetic variants allows a better prediction of the risk of hemolysis, but still leaves unacceptable gaps.

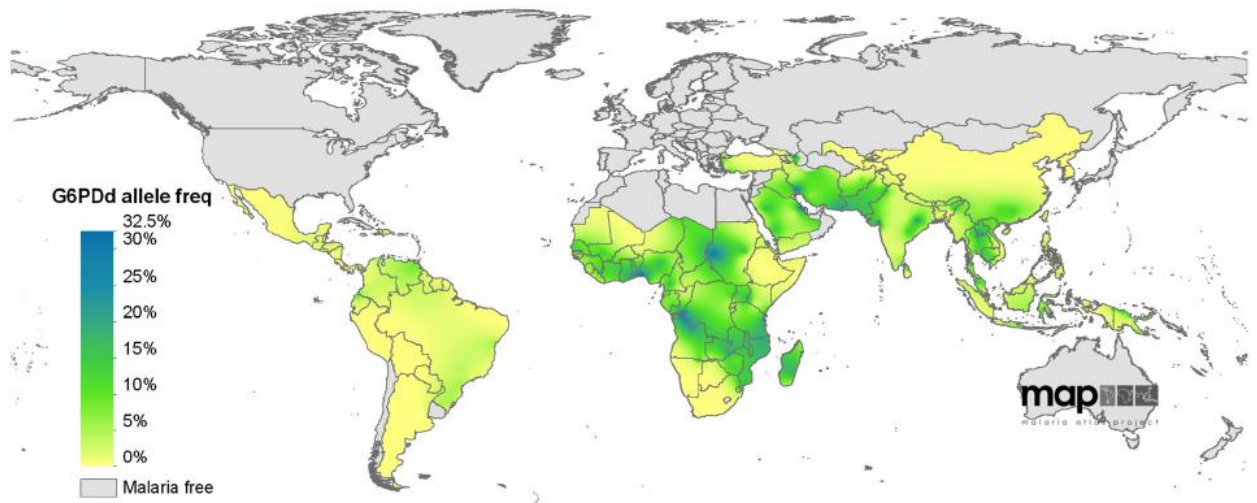


Figure 7. World map of G6PD deficiency in line with malaria endemicity and allelic frequency (122)

4.1.3.4. Genetics and classification of the G6PD deficiency

The G6PD deficiency is X chromosome linked and polymorphic by nature, several mutations are known to occur in close to 400 variants (133). However, the most common variants are few.

The G6PD gene position on the X chromosome has important implications for its population genetics. Unlike in males, for whom the G6PD phenotype is binary, with individuals being either deficient or non-deficient depending upon which allele was inherited (70,126), the gene's X-linked inheritance means that deficiency in females is more complex. Females inherit two copies of the X chromosome and therefore, have two populations of RBCs, each expressing one of the two G6PD alleles they carry. If females inherit two identical alleles (both either normal or deficient), their phenotype and clinical symptoms will be identical to those hemizygous males. Heterozygous females, however, inherit one wild type and one deficient allele, but display a mosaic effect of expression as only one X chromosome is expressed in each cell. One population of cells will express the normal allele and the other population the deficiency (125,134,135). The ratio of normal to deficient cells is variable, due to the phenomenon of lyonization (136). Lyonization is a random process and the resulting proportions of normal and deficient cells may deviate significantly from the expected 50:50 ratio (136), leading some heterozygotes to have virtually

normal expression, and others with expression levels comparable with female homozygotes (i.e. entirely deficient). Heterozygotes may therefore, express a spectrum of phenotypes; making appropriate diagnoses with standard binary methods much harder than for deficient males, as many heterozygotes will be phenotypically normal. At the population level, G6PD deficiency is more commonly expressed in males, though in populations with high frequencies of deficiency, homozygotic inheritance can be common, and the prevalence of affected heterozygotes may also be of public health concern (70,136).

Several classification of the G6PD deficiency are reported by different authors with respective limitations, the discussion of the classification variation is beyond this review, however, the WHO endorsed classification and those below are the most commonly used and accepted ones.

In order to stratify the potential hemolytic risk associated with G6PD deficiency, an index was developed that incorporated both the national prevalence of the trait and the severity of the local genetic variants. The WHO classifies G6PD genetic variants into five classes, the first three of which are deficiency states (137).

- Class I: Severe deficiency (<10% activity) with chronic hemolytic anemia
- Class II: Severe deficiency (<10% activity), with intermittent hemolysis
- Class III: Mild deficiency (10-60% activity), hemolysis with stressors only
- Class IV: Non-deficient variant, no clinical sequelae
- Class V: Increased enzyme activity, no clinical sequelae

Regional patterns in the distribution of variants are reported by several authors (70,70,126,128), the most common variants in human population are G6PD A⁻ and G6PD Mediterranean, followed by Mahidol (70). The sub-Saharan Africa variant G6PD A⁻, is predominantly ranked as mildly severe (class III), although some class II variants were reported from Sudan, South Africa, Senegal and The Gambia (125). Relatively few data are available from the Americas, but these included a greater diversity of variants, including a minority of class II variants. In contrast, variant distribution reports are more heterogeneous across Asia, a majority of which are class II, most

commonly Mediterranean, and certain class III variants (Mahidol, then Chinese-5 and Gaohe being most frequently identified (70).

4.1.3.5. Diagnosis to detect G6PD deficiency

The definitive diagnosis of G6PD deficiency is based on the estimation of enzyme activity, by quantitative spectrophotometric analysis of the rate of NADPH production from NADP (131,132,138). For rapid population screening, several semi-quantitative methods have been applied, such as the dye-decolouration test and fluorescent spot tests, which indicate G6PD deficiency when the blood spot fails to fluoresce under ultraviolet light (70). Other semiquantitative tests have been used, but may require definitive testing to confirm an abnormal result. Several points of care tests are becoming available for G6PD testing. Carestart G6PD kit and Biosensor produced by Access Bio Pharmaceuticals are among the common ones (138,139).

Diagnostic issues can arise for G6PD variants when measuring enzyme activity during an episode of acute haemolysis, or in the presence of a high reticulocyte count, since the level of activity in young erythrocytes is higher than in more mature cells, leading to false negative results for G6PD deficiency (70). None of the rapid screening tests can diagnose heterozygous females reliably, since X-chromosome mosaicism leads to partial deficiency only. Heterozygous females with extremely skewed X inactivation have activity ranging from hemizygote to normal. Molecular analysis is the only method by which a definitive diagnosis can be made of a female's status (140).

In practical terms, testing for G6PD deficiency should be considered when an acute haemolytic reaction follows exposure to a known oxidative drug, infection or foods, such as fava beans, either in children or in adults, particularly if they are of African, Mediterranean, or Asian descent (70). Moreover, members (especially males) of families in which jaundice, splenomegaly, or cholelithiasis are recurrent should be suspected for G6PD deficiency (70).

4.1.3.6. Overlap between malaria and G6PD deficiency endemicity

Malaria and G6PD deficiency share the same geographical distribution and it is thought that G6PD deficiency may be protective against malaria (141,142). G6PD deficiency similar to other human RBC polymorphisms is particularly prevalent in historically malaria endemic countries. The spatial extent of *P. vivax* malaria overlaps widely with that of G6PD deficiency (125,128).

The spatial overlap between G6PD deficiency and the pre-control distribution of malaria was stated by Motulsky and Allison shortly after the description of G6PD deficiency (125). G6PD deficiency carried, at least in some genotypes (homo- or heterozygotes), a selective survival advantage over normal enzyme levels against malaria morbidity or mortality. This has attracted much attention over the past half a century with evidence from epidemiological observations, *in vitro* laboratory finding studies and *in vivo* clinical studies strongly supporting the hypothesis that, this common genetic trait has been selected by malaria through conferring some degree of resistance against the severity of the infectious diseases (70,128).

The most convincing epidemiological evidence of selection by malaria is the diversity of variants of the G6PD gene, many of which have reached polymorphic frequencies in genetically isolated populations, suggesting the independent selection of each variant. Micro-mapping studies across altitudinal or climatic gradients of malaria endemicity also suggest that the prevalence of this disorder is the result of selection by malaria, rather than random drift or selection (128,135).

4.1.3.7. G6PD deficiency in the context of malaria elimination

Efforts for the global eradication/elimination of malaria have been undertaken by the WHO approximately 50 years ago, (122). Currently, with similar initiatives several countries have re-started malaria elimination efforts. As stated before, the depletion of the parasite reservoir in the human host requires detection of both symptomatic and asymptomatic individuals and killing all forms of the parasite that they carry (30,82). Primaquine is the only recommended drug by WHO in elimination settings (69,74,134) and the related 8-aminoquinoline Tafenoquine is gaining momentum given that it can be administered as a single dose regimen, but with a risk of haemolysis

in G6PD deficient individuals (69,74). Tafenoquine was recently approved by the United States Food and Drug Administration (FDA) (69). Both drugs can cause haemolysis in individuals who are deficient in G6PD, with increasing risk when high dose regimens are used. The recently introduced short course (7 days) Primaquine, although it may have a relative advantage of increasing adherence over the 14 days course, may carry a similar risk of haemolysis in deficiency communities (76). Even low dose regimes (0.35mg/kg x 14 days) in individuals with mild G6PD deficiency can induce transient haemolytic episodes (73,143). There is a strong need for high-quality rapid point of care tools pretreatment G6PD tests, for the reliable detection of G6PD deficiency prior to treatment. .

4.1.3.8. G6PD deficiency in Ethiopia

Until recently, the issue of G6PD deficiency in Ethiopia has been infrequently discussed and its implications on malaria control program have been limited. The malaria control program in Ethiopia was using Primaquine during the malaria Eradication Period (1955-1969) (122,144). During that period, Ethiopia, due to its low to moderate malaria transmission has been considered appropriate for malaria elimination, and large scale efforts for malaria elimination were implemented. Since that period, Primaquine has been used for the treatment and radical cure of vivax malaria, without G6PD testing. There are no accessible data which would document hemolysis resulting from the use of Primaquine. The use of Primaquine was stopped with the introduction of Sulfadoxine Pyrimethamine (Fansidar) for the treatment of *P.falciparum* in 1999 (145–147). But the current literature review did not find a documented reason for the termination of Primaquine use. Both *P. falciparum* and *P. vivax* were treated using a combined Chloroquine and Primaquine until the introduction of Sulphadoxine Pyrimethamine. Personal communications indicated that with *P. falciparum* developing resistance, Chloroquine was replaced by Sulphadoxine Pyrimethamine. *P. vivax* remained sensitive to Chloroquine (148), the importance of combination treatment was reduced. Additionally, as the GMEP terminated and Ethiopia returned back to aim for control, radical cure was considered less important, except exposing individuals to potential toxicity, thus Primaquine use was deferred indefinitely (Tesfaye Mengesha, personal communication).

Review of literature from the 1960s, showed several G6PD deficiency studies in Ethiopia, made around the high lands or in selected low land areas of the country (117,132,140,141,149–152). There is no nationally representative study on the prevalence and distribution G6PD deficiency to date. These isolated studies documented the absence of the deficiency in the major ethnic groups, with G6PD deficiency prevalence ranging from 1.4 – 14.3% reported in the country's southwest (Nuer and Anuak) and northeast (Aregoba in Afar) (132,141,149,153). Similar study conducted in Israel on persons of Ethiopian origin showed the absence of the deficiency (151). The communities were living in the northern highland of Ethiopia before they moved to the State of Israel (154).

However, considering the geographical position and the ethnic mix of Ethiopia, the G6PD deficiency prevalence cannot be ruled out, it may be prevalent in parts of the country where malaria is endemic. Hence, there is a need for overall representative G6PD deficiency study, inclusive of the western lowlands of the country, where malaria is endemic and local communities are phenotypically/genetically different.

The current thesis reported the genotypic prevalence of common G6PD deficiency allelic types in the general population residing in malaria-endemic areas of Ethiopia.

5. CONCEPTUAL FRAME WORK

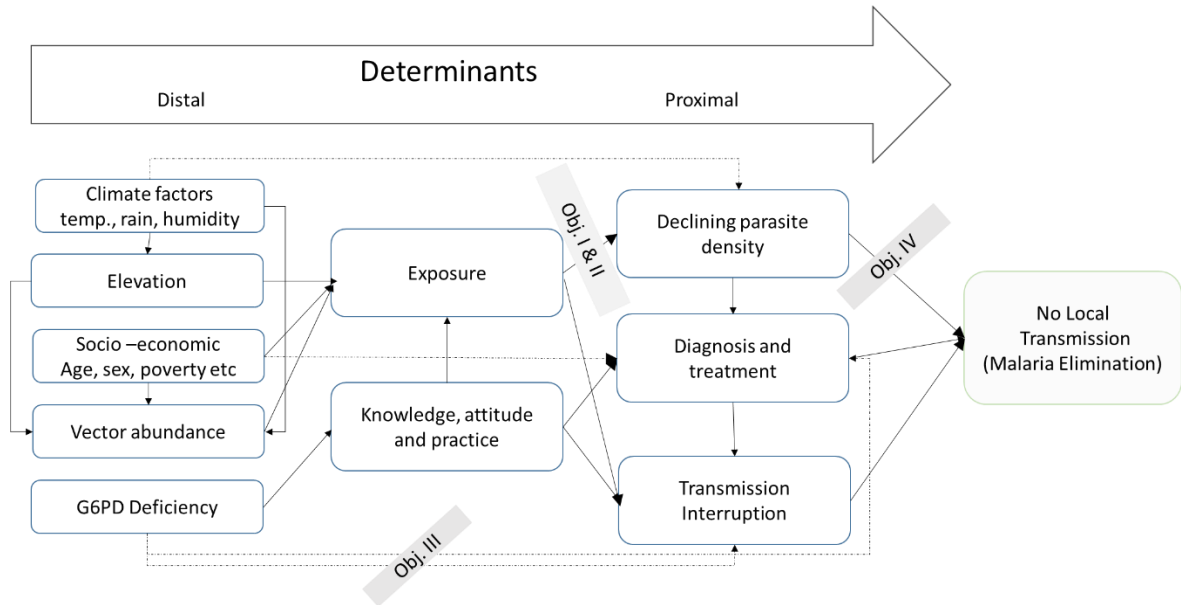


Figure 8. Conceptual Framework of the study:

The conceptual framework of the study is shown in figure 8. The diagram depicts part of the interrelated determinants in malaria elimination. Considering no local transmission as the outcome of malaria elimination, the proximal/immediate determinants are listed in the right side of the diagram; followed by the individual and community level indicators and the ones on the far left of the diagram are distal, but important determinants that have indirect effect on malaria elimination. This may include, environmental factors, socioeconomic factors and some other non-modifiable factors, such as genetic abnormalities that complicate malaria elimination efforts. The strategic position of the four objectives are indicated on the diagram.

6. OBJECTIVES

6.1. General objective

The aim of this study was to generate new epidemiological evidence for the agenda of malaria elimination in Ethiopia; using advanced molecular and diagnostic tools and nationally representative samples collected in 2011 and 2015.

6.2. Specific objectives

1. To estimate national and regional seroprevalence in Ethiopia
2. To determine transmission pattern of malaria in Ethiopia
3. To determine low density parasitaemia prevalence using sub-national samples
4. To determine G6PD deficiency and allelic types in malaria endemic areas of Ethiopia

7. MATERIALS AND METHODS

7.1. Study area and population

The study was conducted as part of the National Ethiopian Malaria Indicator Surveys (EMIS). Ethiopia has successfully conducted three national malaria indicator surveys, in 2007, 2011 and 2015 (55,60,61). The National Malaria Control Program (NMCP) at the Federal Ministry of Health (FMOH) categorizes the Country into malaria free, low, moderate and high transmission areas based on malaria transmission intensity and elevation. Each category receives tailored malaria control activities that fits the situation. The malaria control activities, include vector control, case management and surveillance (53). EMISs have been conducted to measure the coverage and utilization of malaria control interventions, using malaria intervention indicators in malaria transmission areas (55,60,62). Generally, areas below 2000 meters above sea level (masl) are considered to be malaria endemic. Areas between 2000 and 2500 masl are malaria fringe areas and prone to malaria transmission and epidemics (53,155). More than 60% of the over 100 million people in Ethiopia live in malaria risk areas. The sampling design for EMISs provided national estimates for malaria endemic and malaria prone areas of the country (Figure 9), this represent the study area and population for the current study. Figure 9 shows the sample collection sites for EMIS 2011 and EMIS 2015 in Ethiopia. Each dot represent the cluster of 5-25 houses. The study samples were collected during the major malaria transmission seasons, between September and December in the respective years.

Samples collected together with EMIS 2015 were used to address objectives 1, 2 and 4 and sub-samples collected together with EMIS 2011 were used to address objective 3 of the current study.

A**B**

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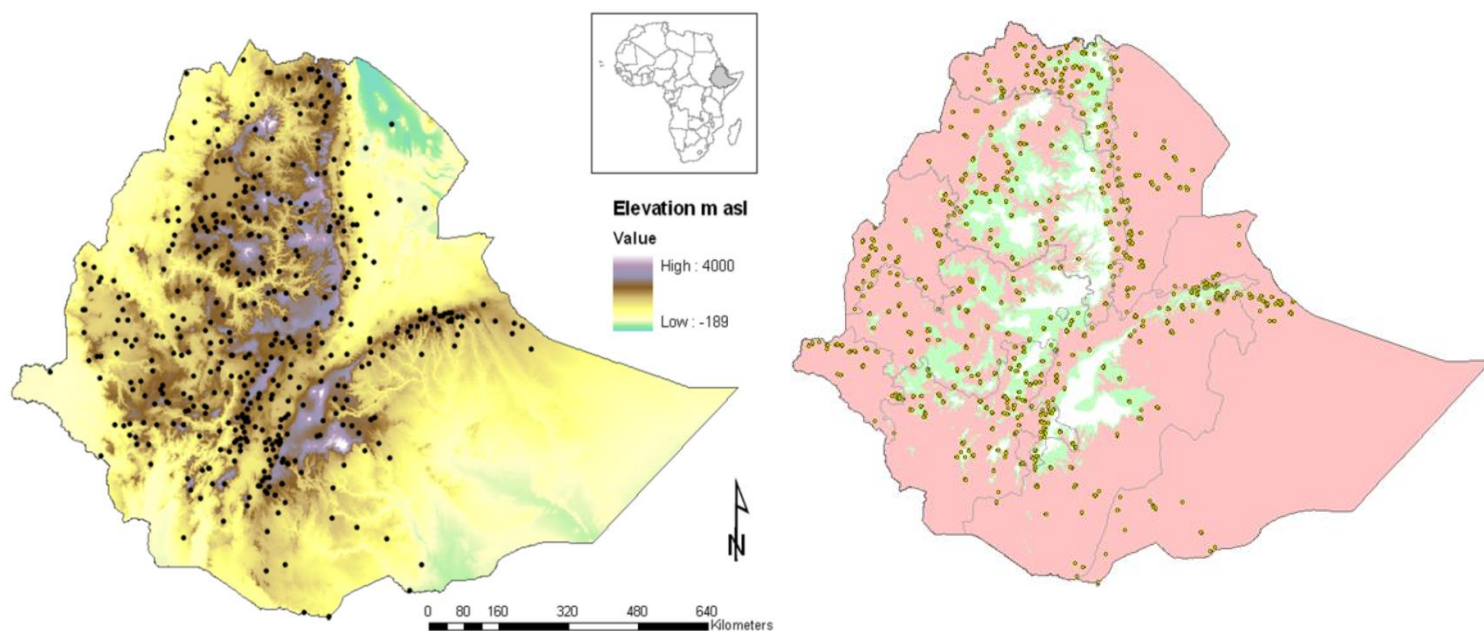


Figure 9 : Geographical distribution of sample collection household's. A, Malaria indicator Survey 2011 (EMIS-2011). B, Malaria Indicator survey 2015 (EMIS-2015). Darker area in figure A and lighter area in Figure B show the central high lands compared to the lowlands.

7.2. Study design

The study was a cross-sectional household survey that used a representative probability sampling technique to produce national and sub-national estimates of major malaria intervention indicators for malaria-endemic (less than 2000 masl) and malaria prone areas (above 2000 and less than 2500 masl) across Ethiopia. It had several domains of estimates stratified based on elevation, socioeconomic and political boundaries. The level of estimation included: 1) National for areas less than 2000 masl and national for areas above 2000 masl and less than 2500 masl, 2) sub national for areas less than 2000 masl (Afar, Amhara, Benishangul-Gumuz, Dire Dewa, Gambela, Harari, Oromia, Southern Nations and Nationalities People's (SNNP), Somali and Tigray Regions).

The study followed two-stage cluster sampling methodology. In the first stage, enumeration areas (EAs) were selected with probability proportional to EA size. An EA is a unit of land delineated for the purpose of enumerating housing units and population; used during national population census. An EA usually consists of 150–200 households. The required sampling frame of EAs was provided by the Ethiopian Central Statistics Agency (CSA). The second sampling was made from the onsite listing of all households in the selected EAs and 25 households were randomly selected.

A single population proportion formula was used to determine the minimum sample size required for the survey per domain:

$n = ((Z^2 \times P \times (1-P) / d^2) \times (DEFT)^2$, where: n is the required minimum sample size of households, P the expected proportion (explained below), d is the absolute precession, (calculated as $((2 \times P \times RSE)/100)$, RSE is the Relative Standard Error (relative precession) of the expected proportion, and DEFT is the design effect accounting for the two-stage cluster sampling method (Annex 3).

During EMIS 2011, the sample size was determined using proportion of Indoor Residual Spraying (IRS) coverage as the key indicator (20%) (55), a relative precession of 12%, 95% confidence limits, 80% power, a design effect of 1.25 (established based on an earlier similar survey), and 20% adjustment for non-response (from household refusals, or abandoned households). The sample size assumes that 82% of households have children of under-five years of age. Based on the assumptions, a minimum sample size of 7,725 households was calculated to obtain national-level estimate for elevation below 2,500 masl and urban and rural level estimate for elevation below 2,000 masl. To satisfy the specific programmatic needs over sampling was made to four major regions (Oromia, SNNPR, Tigray, and Amhara Region) and combined over sampling was made for Afar/Somali regional states and Benishangul-Gumuz/Gambella regional states. An additional 3,375 households were added from the overestimate to give a total sample size of 11,100 HHs in 444 EAs (EMIS 2011).

In EMIS 2015, the major key indicator used to calculate the samples size was the proportion of under five children who slept under a mosquito net the previous night from EMIS 2011 (38.2%), relative precession of 9%, 95% CI, design effect of 2, 72% of households have children under five (Data from EMIS 2011), and 20% non-response rate. Based on the assumptions formula, the sample size required per domain was 1,388 House holds (HHs). Since the 2015 EMIS was

supposed to produce regional level estimates, the total sample size for the country was obtained multiplying by the ten malarius regions 13,880 (1,388X10) HHs in 555 EAs (EMIS 2015).

The major reason for the difference of sample size among the two surveys was due to the level of estimation considered during EMIS planning. EMIS 2015 assumed separate estimates to all the nine regional states and Dire Dawa town. This was in order to accommodate the programmatic need of each region. Whereas in EMIS 2011 separate estimates were made for the major four regions (Amhara, Oromia, SNNPR and Tigray regions) and aggregate estimation was assumed for Afar and Somali and Benishangul Gumuz and Gambela regions. There was no separate estimate considered for Dire Dawa and Harari Regions. Convenience and limited resources were the reasons to make an aggregated estimation during EMIS 2011.

7.3. Sub-sampling of the EMIS-2011 samples

Of the 11,100 DBS samples collected during EMIS 2011, a sub sample of 1,921 was selected by random sampling, assuming a sample proportion of 10%, 95% confidence interval with margin of error equal to 0.015 and adjusted for 20% for lost and poor quality samples ($n = ((Z^2 \times P \times (1-P) / d^2))$).

7.4. Data collection

In both surveys, around 300 personnel were involved in the data collection. The surveyors were government employees on the health sector (health officers, nurses, laboratory technicians and biologists), recruited proportionally to the region size through regional health offices. The data collectors were trained in data and sample collection (see below) for two weeks and organized into 34 teams. Each team had four pairs of a laboratory technician and clinician (Nurse or Health officer), a team leader, and a driver. They were equipped with digital data collection tools (smart phones or Personal Digital Assistant (PDA)) with GPS capability, reagents and materials used in diagnosis and sample collection. Hands on training was provided on delineating an EA, taking geographical coordinates, completing questionnaire, using digital devices, diagnosing malaria,

using Rapid Diagnostic Tests (RDTs), measuring hemoglobin, collecting Blood Films (BF) for microscopy and Dried Blood Spots (DBSs). The author of this dissertation coordinated both surveys and played a key role in the overall leadership, developing training materials, training data collectors, data collection, supervision, data cleaning, data analysis and write up of the final reports.

As indicated in section 5.2, the EMIS, followed the standard two-stage cluster sampling methodology, where by in the first stage EAs were selected with a probability proportional to size from a stratified sampling frame assigned to domains defined by the Federal MoH and Ethiopian Central Statistics Agency (CSA). CSA constructed the sampling frame from the list of EAs used for the 2010 national census. Attempt was made to include EAs with potential malaria transmission by including EAs with an elevation of below 2500m (Annex 3). The second stage sampling was the selection of HHs from each allocated EA, it was conducted at the time of fieldwork using Personal Digital Assistants (PDAs) or smart phones with global positioning system (GPS) capability. Active census of HHs were made and twenty-five households were selected by simple random sampling. In each selected household, a questionnaire was administered to the household head and all women of reproductive age (taken as ages 15-49 years). Samples were collected from all children under 5 years of age and all family members in every 4th household.

7.5. Biological sample collection

Biomarkers in the EMIS included diagnosis of malaria and anaemia on the spot, using RDT and portable spectrophotometer (Hemo-Cue®), respectively; and collection of blood film and DBS for later analysis. All samples were collected from a single finger prick (capillary blood) from consented persons and care takers, in case of children.

For malaria diagnoses, thick and thin blood smear were prepared to be stained later in the evening in the near by health center. Further 2-3 blood drops were applied to a Whatman 3 filter paper. The blood spots were dried on the spot, labeled and packed in sealable bags with a desiccant. The samples were temporarily stored in freezers in the nearby health centers and later transferred to the Ethiopian Public Health Institute (EPHI) to be stored at -20⁰ C until use.

All DBS samples collected and available were assayed for antibody response (see section 5.7.1). Based on the serology results, all samples from Tigray, Amhara and Benishangul Gumuz regions were selected to represent low to high malaria transmission and were assayed for sub-patent infections. Sub-samplings were made to select representative samples for G6PD study. The sub samplings were implemented due to resource constraints and the complexity of the studies.

7.6. Laboratory methods and measurements

7.6.1.1. Multiplex Serology

An advanced version of Enzyme-Linked Immunosorbent Assay (ELISA) was used in the current study. Antibodies were eluted from dried blood spots, and samples were assayed for Immunoglobulin G (IgG) against *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale* antigens. Data were collected in Multiplex Bead Based Assay using a MagPix instrument (Luminex Corporation, Austin, TX). Previously published methods were followed in laboratory analysis (42,156).

7.6.1.2. Blood spot elution

A 6 mm punch was used from each DBS sample and blood eluted to a 1:20 concentration in blocking buffer (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) overnight. Eluate was stored at 4°C using round bottom 96 well plates, until further analysis.

7.6.1.3. Antigens

Six *Plasmodium* antigens were used for this study: two *P. falciparum* antigens (Merozoite Surface Protein-1 (MSP-1) and Apical Merozoite Antigen-1 (AMA-1)), two *P. vivax* antigens (MSP-1 and AMA-1), one *P. malariae* (MSP-1) and one *P. ovale* (MSP-1) were investigated. The four

Plasmodium MSP-1 19kD antigens were recombinantly produced and purified as described previously (156). The external domain of AMA-1 antigen was produced at Walter Reed Army Institute of Research (WRAIR) under previously published conditions (43). The *P. vivax* AMA-1 antigens were produced at London School of Hygiene and Tropical Medicine (LSHTM) under previously published conditions (157). The *Schistosoma japonicum* glutathione-S-transferase (GST) antigen was produced recombinantly and served as a generic protein to assess IgG non-specific binding.

7.6.1.4. Bead coupling

All antigens were coupled to magnetic beads (Luminex Corporation, Austin, TX) in the same manner as prior studies (42,156). Briefly, beads were pulse vortexed, transferred to a microcentrifuge tube and centrifuged for 1.5 minutes at 13,000g. Supernatant was removed and beads were washed with 0.1M sodium phosphate, pH 6.2 (NaP). Beads were activated by suspending in NaP with 5 mg/mL of EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) and 5 mg/mL sulfo-NHS (sulfo N-hydroxysulfosuccinimide) and incubating with rotation for 20 minutes at room temperature (RT) protected from light. After a wash with coupling buffer (50 mM 2-(4-morpholino)-ethane sulfonic acid, 0.85% NaCl at pH 5.0), antigens were coupled to beads in presence of coupling buffer for 2h at an antigen concentration of 20 ug/mL for all antigens, except for PvAMA1 and GST at 15ug/mL. Beads were washed once with Phosphate buffered saline (PBS), and suspended in PBS with 1% bovine serum albumin (BSA) with incubation for 30 minutes at RT by rotation. Beads were then re-suspended in storage buffer (PBS, 1% BSA, 0.02% sodium azide and 0.05% Tween-20) and stored at 4°C.

7.6.1.5. Bead based assay

The DBS elution was assayed for IgG antibodies using bead-based multiplex technology and the One Step immunoassay protocol as described previously (156). In 5mL reagent buffer (Buffer A: PBS, 0.5% BSA, 0.05% Tween-20, 0.02% NaN₃), a bead mix was prepared with all coupled bead

regions included, and 50uL bead mix was pipetted into each well of a BioPlex Pro plate (BioRad, Hercules, CA). Beads were washed two times with 100uL PBST, and 50uL reagent mix [in 5mL Buffer A: 1:500 anti-human IgG (Southern Biotech), 1:625 anti-human IgG₄ (Southern Biotech), 1:200 streptavidin-PE (Invitrogen)] was added to all wells, then 50uL samples (or controls) were added to the appropriate wells at a sample dilution of 1:50. Plates were incubated overnight with gentle shaking at RT and protected from light. The next morning (after ~16h total incubation time), plates were washed three times, and beads re-suspended with 100uL PBS and read on a MAGPIX machine. Mean fluorescence intensity (MFI) signal was generated for a minimum of 50 beads/region, and background(bg) MFI from wells incubated with Buffer B was subtracted from each sample to give a final value of mean fluorescence intensity subtracted background (MFI-bg).

7.7. Determination of subpatent infection

7.7.1. Sample source

Due to limited resources, samples from three regions were used for this study, namely from Amhara, Benishangul Gumuz and Tigray Regions (Figure 10). The geographical areas represent very low to moderate/high malaria transmission settings based on serological data analysed in this study. Patients diagnosed by malaria microscopy and RDTs that have provided DBS samples in the three regions were selected among EMIS 2015 samples depository at the Ethiopian Public Health Institute (EPHI), where samples are stored at -20°C.

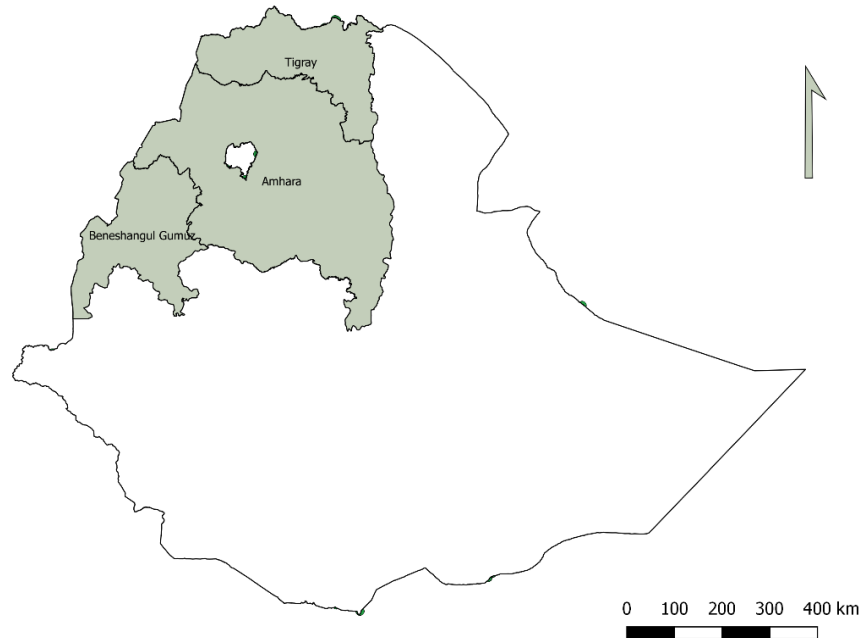


Figure 10. Administrative regions where samples selected for subpatent study (Tigray, Amhara and Benishangul Gumuz)

7.7.2. Microscopy and RDT tests

Thick and thin blood smears were read by WHO certified microscopists. The slides were stained with 3% Giemsa for 10 min and screened for the presence of plasmodial infections. Microscopists were blinded to the RDT results. A slide was classified as negative if no *Plasmodium spp* asexual forms or gametocytes were found after viewing 100 fields oil immersion objective. Any positive slide was confirmed by two other microscopists. CareStart™ tests for histidine-rich protein 2 (HRP 2) to detect *P. falciparum* and pan-*Plasmodium* lactose dehydrogenase (LDH) for *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae* was used. The CareStart™ Malaria HRP2/pLDH (Pf/PAN) Combo RDT was used to detect *Plasmodium falciparum*/ *Plasmodium* species in the field.

7.7.3. Polymerase Chain Reaction (PCR)

Nested PCR was used to detect the four *Plasmodium* species (*P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*). The laboratory work was carried out at the Malaria Branch Laboratory at the Centers for Disease Control and Prevention, Atlanta. Selected DBSs were shipped at ambient temperature.

Two-step pooling was used to estimate prevalence of malaria in RDT negative samples, as previously described by Zhou et al., (38). A pilot was carried out to see the effect of age (pooled by age >5 and <5) and pool size of 5 or 10 individual samples, no significant difference by age or pools size was found.

Six millimeter disk was punched out from each DBS card and ten disks were pooled together for DNA extraction. Pooled DBSs were extracted with QIAGEN Mini DNA kit as per the manufacturer's protocol (QIAGEN, Valencia, CA) with slight modification. Samples were left for an overnight incubation in ATL buffer plus Proteinase K to ensure proper lysis during pooling. Then, samples were extracted following a normal extraction protocol. The purified DNA were tested using nested genus PCR (nPCR). For any pool with a positive PCR result, individual DBSs were extracted following the same protocol as the pool samples and species-specific PCR assays performed.

Of the total 2669 samples available, 61 samples were excluded due to lack of complete data. Seventy three RDT positive samples were analyzed and confirmed by PCR individually. The remaining 2,533 RDT negative samples (945 from Amhara, 668 from Benishangul Gumuz and 922 from Tigray Regions) were available for pooling. (Figure 11).

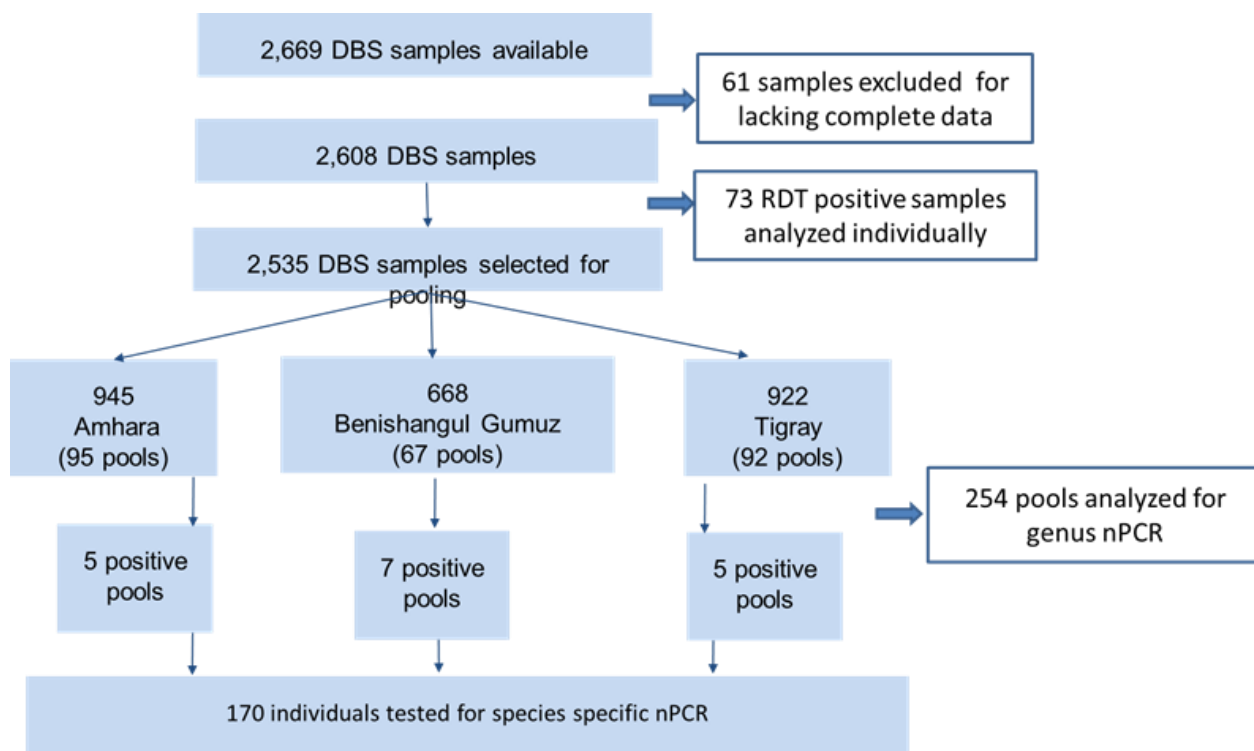


Figure 11. Flow chart showing sample pooling and PCR analysis approach

7.7.4. nPCR detection for ribosome RNA (rRNA)

Purified DNA templates were used for amplification of the 18S rRNA gene subunit using a modified method as previously described (38). Briefly, the 50 µl PCR reaction contained 1× PCR Master Mix (Promega, Madison, WI) and 400 nM of each PCR primer. For each primary PCR reaction, 5 µl of DNA were used. The reactions were performed under the conditions of initial denaturation at 95°C for 5 min, 25 cycles at 94°C for 1 min, annealing at 58°C for 2 min, and final extension at 72°C for 5 min. Secondary PCR was performed as primary PCR, except for use of 35 cycles and 2µl primary PCR product per reaction. The amplified DNA products were stained with GelRed (Biotium, Hayward, CA) in 2% agarose gel electrophoresis and visualized under UV illumination.

7.8. Determination of G6PD allelic types

7.8.1. Restriction Fragment Length Polymorphism

A standard Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) technique was used for DNA genotyping. Based on a review of the literature, the country's geographic proximity to the Arabian Peninsula and Ethiopia's ethnic mix, the following three G6PD mutations common in Africa and the Mediterranean regions were selected for genotyping: G6PD*A (A376G), G6PD*A- (G202A) and Mediterranean (C563T) (128,140,158).

PCR-RFLP was based on previous studies (131,159), and the annealing temperature was modified to reduce nonspecific amplification. DNA was extracted from DBS samples, using the Qiagen DNA mini-kit (Qiagen, Germantown, USA). PCR was used for the amplification of three specific regions. DNA was first amplified using primers for G6PD*A (A376G), 5'-CCCAGGCCACCCCAGAGGAGA-3' (forward) and 5'-CGGCCCCGGACACGCTCATAG-3' (reverse) and all samples positive for G6PD*A (A376G) were then subjected to PCR amplification, using primers for G6PD*A- (G202A), 5'-CACCACTGCCCCTGTGACCT-3' (forward) and 5'-GGCCCTGACACCACCCACCTT-3' (reverse). All DNA extracts were also amplified for Mediterranean (C563T) type mutation using primers 5'-AGCTCTGATCCTCACTCCCC -3' (forward) and 5'- GGCCAGGTGAGGCTCCTGAGTA -3' (reverse). The following PCR conditions were used for 376 and 202 G6PD mutations: initial denaturation of one cycle for 5 minutes at 94°C, then 32 cycles for denaturation of 45 seconds at 94°C, annealing of 30 seconds at 64°C, extension of 45 seconds at 72°C, and final extension at 72°C for 1 min. Similar PCR conditions were used for the Mediterranean (C563T) mutation with the following modifications: initial denaturation of one cycle for 5 min at 94°C, then 32 cycles for denaturation of 1 min at 94°C, annealing of 1 min at 57°C, extension of 1 min at 72°C and final extension at 72°C for 1 min.

7.8.2. Restriction enzymes digestion

Three restriction endonuclease enzymes, FokI (G6PD*A (A376G)), NlaIII (G6PD*A- (G202A)) and MboII (Mediterranean (C563T)) (New England Biolabs Ltd, Ipswich, MA), were used for digestion of the amplified products (Table 2). Samples were incubated with restriction enzymes for 1 hour at 37°C, followed by inactivation at 65°C for 25 minutes (131,159–161). Final PCR-RFLP products were separated using 2% agarose gel-electrophoresis and visualized under UV illumination.

Known blood samples archived at CDC, collected from a cross-sectional survey in Kenya and a malaria in pregnancy project in Malawi, served as positive controls for G6PD*A (A376G) and G6PD*A- (G202A) (African type mutations). However, there were no positive controls used for the Mediterranean (C563T) type mutations. Results with faint and intermediate bands were repeated using the concentrated DNA samples to achieve more distinct test results. The testing was conducted at the Malaria and other Parasitic Diseases Research Unit at the Ethiopian Public Health Institute (EPHI). A sub-set of samples (10%) were also genotyped at the CDC, in Atlanta for training and quality assurance testing.

Table 2 Types of G6PD variants screened by RFLP and expected size of fragments in base pairs.

Mutation	Variants	Restriction enzymes	Size	Fragment Size		Reference
				wild type	Mutant	
	G6PD B→G6PD					
376 A→G	A	FokI	308	308	125, 183	Samilchuk et al. 1999(162)
						Samilchuk et al. 1999(162)
202 G→A	A ⁻	NlaIII	211	211	81, 130	Alfadhli et al. 2005(163)
563 C→T	Mediterranean	MboII	285	33, 252	33, 98, 154	

7.9. Data analysis

EMIS data were available in Microsoft Access (EMIS-2011) and Microsoft Excel formats (EMIS-215), and additional data generated were entered into Microsoft Excel. Data were exported to STATA and SAS for further analyses.

7.9.1. Serology data analysis

Data generated in Magpix bead based assay were imported into an Excel spreadsheet for cleaning, further analysis was made using Stata 13 (College Station, USA). GST (antigen extracted from *Schistosoma japonicum*) reads (MFI-bg) above 1000 MFI value were excluded from analysis, as it shows nonspecific binding. To dichotomize seropositivity, log-transformed MFI-bg values were fitted in to a two-component Finite Mixture Model (FMM) by the FMM procedure with normal distribution and maximum likelihood estimation outputs. A seropositivity cutoff value was determined by the mean MFI-bg value of the first (assume seronegative) component plus three standard deviations (156,163). Overall, *Plasmodium falciparum* and *P. vivax* seropositivity was defined as an individual being positive for either or both of the MSP-1 and AMA-1 antigens. Species specific MSP-1antigens were used for *P. malariae* and *P. ovale* seropositivity. To minimize cross-reactivity between the species-specific MSP antigens, an additional conservative two-stage approach that may increase specificity, but decrease sensitivity of the specific antibodies to the targeted antigens is reported. This conservative approach may avoid the bias on potential cross reactivity among the orthologs. For this conservative approach for *P. malariae* and *P. ovale*, individual readings for PmMSP-1 or PoMSP-1 response first had to be above the MFI-bg cutoff as described above. Additionally, the PmMSP-1 and/or PoMSP-1 MFI-bg signal for that sample also needed to be above the PfMSP-1 MFI-bg signal for the same sample (ratio to PfMSP-1 greater than 1.0) to be considered PmMSP-1 and/or PoMSP-1 positive.

National and regional prevalence was estimated for each species. Sampling weights were calculated during MIS 2015 to weight the data and ensure the representativeness of the samples tested to the study population. Adjustments were made by region, elevation, and age group. Linear

and multiple logistic regression models were employed to determine odds ratios [OR], with 95% confidence intervals [CI] for the effects of sex, age and elevation. Similar regression models were used for *P. falciparum* and *P. vivax* seropositivity.

A reversible catalytic model was fitted to the dichotomized data using maximum likelihood methods to generate a seroconversion rate (SCR or λ) and a seroreversion rate (ρ). The model was used to generate age seroprevalence curves, from which a seroconversion rate (SCR) representing the force of infection for the community was calculated. Evidence for two forces of infection was investigated, if visual inspection of SCR curves indicated such a comparison and was guided by a profile likelihood plots to determine the most likely time (year) of change in transmission (80, 84).

7.9.2. Sub-patent infection data analysis

Sub patent infection was defined as a *Plasmodium spp* infection detected in study participants with infection densities too low to be detected by microscopy and malaria RDT, but detectable by more sensitive tools, such as PCR (131). Descriptive statistics with 95% confidence interval were estimated for demographic characteristics. Multiple logistic regression was used to assess the association of risk factors (sex, age, elevation, bed net use and seropositivity) with nPCR parasitemia results. Percent relative difference and concordance for microscopy and RDTs were calculated against PCR results as the reference standard. Values were considered significant at $P < 0.05$. Venn diagram was constructed using STATA to visualize the relationship amongst the four diagnostic methods: microscopy, RDTs, nPCR and serology.

7.9.3. G6PD deficiency data analysis

Laboratory results (gel readings) were interpreted as being either wild type, hemizygous, homozygous or heterozygous allelic types. National and regional frequencies were calculated for each mutation observed with 95% confidence interval. To ensure the representativeness of the samples, sampling weights were calculated by performing a weighting class adjustment to force

weights from those tested for G6PD sub-sampling to be equal to the total weight in the full sample. Adjustments were made by region, altitude, and age group (165).

7.10. Spatial analysis

Spatial analysis and geographical mapping were done using QGIS version 2.1.8 and ArcGIS version 10.5 (ESRI, California, USA) software's. Spatial distribution of samples were geocoded, for 2011 and 2015 samples using QGIS. Heat maps for seroprevalence data were developed by kriging methods in ArcGIS. Empirical Bayesian kriging was used to predict the spatial distribution of seroprevalence as a continuous surface of probability being above a cut-off (mean seronegative +3sd). This is an established method which uses a statistical model to predict and interpolate the spatial distribution of a variable from available data (166). Average seroprevalences per EA were plotted using household coordinates. Maps were developed separately for MSP-1 and AMA-1 antigens for both *P. falciparum* and *P. vivax* for all individuals and for children under five years of age. QGIS version 2.1.8 and ArcGIS version 10 were used.

7.11. Ethical considerations

The PhD proposal received ethical clearance from the College of Health Sciences, Addis Ababa University (AAUMF 03-008) and received non-research determination from the Centers for Disease Control (CDC), Atlanta. (Human Subjects Research Tracking # 2015-244).

Verbal informed consent was sought from the heads of households and each eligible individual in accordance with the tenets of the Declaration of Helsinki. Verbal informed consent was sought from each eligible individual and parents of children under five for blood samples. Additional verbal informed assent was sought from children aged 8-18 years. Consent forms used are presented in Appendix 6.

All individuals confirmed to be positive by RDTs were provided treatment according to the FMOH, National Malaria Treatment Guidelines (167–169). This was based on Artemether-Lumefantrine

(CoArtem®) for *P. falciparum* infection and Chloroquine for other *Plasmodium* species infections. Referral linkage was insured for clinic based quinine therapy for self-reported pregnant women. People who were found to be malaria-negative by RDT, but febrile or otherwise ill, were referred to the nearest health facility for further investigation.

For children diagnosed with moderate to severe anemia (i.e. hemoglobin >5 and ≤ 8 g/dl), results were shared with the parent/guardian and the children were referred to the nearest health facility. If a parent/guardian refused to visit the health facility, children were given Artemether-Lumefantrine (170), Albendazole as per the National Protocol for Integrated Newborn and Child Illnesses (171) and a two-week supply of supplemental iron.

Children with severe anemia (i.e. hemoglobin ≤ 5 g/dl) were referred to the nearest health facility for further evaluation and management. Subjects who were found to be severely ill, as determined by the survey nurses, were immediately escorted to the nearest possible health facility for further evaluation.

The MIS survey protocols received ethical clearance from the Ethiopian Public Health Institute (EPHI), Ethiopian Science and Technology Agency, and the study was also reviewed and approved by the following international collaborators: Emory University Institutional Review Board, the U.S. Centers for Disease Control and Prevention Institutional Review Board, and the PATH Ethical Committee. The study was identified by the committees as a non-research determinant, with minimum ethical concern.

7.12. Dissemination of results

The study results were communicated to the Ethiopian Ministry of Health in the form of technical reports, to be used for evidence based decision. Three publications emanating from the study are submitted to peer reviewed journal (Malaria Journal). Two papers are already published (Objective I, II and III), and the third is currently under review by the date (objective IV).

The study findings were presented in several national and international scientific conferences. Nationally: to the 27th and 30th Ethiopian Public Health Association Annual conferences (EPHA 2016 and 2019), 4th Ethiopian Public Health Institute Science Congress (EPHI 2019), World Malaria Day, Axum (2016). 11th National Malaria Research Network Symposium, Debre Markos (2019). Internationally: to the 65th and 67th American Society of Tropical Medicine and Hygiene annual conferences, United States (ASTMH 2016 and 2018) and 14th International Conference of Parasitology, South Korea (ICOPA, 2018). The PhD work will be defended publicly and the monograph will be archived at the AAU both in electronic and printed formats.

8. RESULTS

The results follow the presentation order of the methodology where, findings for malaria seroepidemiology, subpatent malaria prevalence and glucose six phosphate dehydrogenase (G6PD) allelic types and distribution described in the respective order.

This thesis included a number of technically different, but interrelated studies that may help further strengthen and accelerate the malaria elimination efforts in Ethiopia. The main findings of the study are summarized in Table 3 below followed by subsequent details.

Table 3 .Concise Summary findings of the different studies in the thesis, 2015-2019.

Objectives	Papers	Summary findings
1. Estimate national and regional seroprevalence in Ethiopia	II	✓ National seroprevalences were: 32.1%, <i>P. falciparum</i>, 25.0%, <i>P. vivax</i>, 8.6% <i>P. malariae</i> and 3.1% <i>P. ovale</i> ✓ By region: <i>P. falciparum</i> ranged, from 11.0% in Somali to 65.0% in Gambela and <i>P. vivax</i> 4.0% in Somali to 36.7% in Amhara
2. Determine transmission pattern of malaria in Ethiopia	II	✓ For <i>P. falciparum</i> seroprevalence estimates were higher at lower elevations compared to higher elevations. The opposite was true for <i>P. vivax</i> ✓ Higher seroprevalence was observed in the west compared to the east of the country ✓ Seroconversion rates varied by elevation and regions and antigen type
3. Determine low density parasitaemia prevalence	III	✓ DNA screening by nPCR detected 3.3% positive cases ✓ nPCR detected additional 30 samples that were not detected by conventional methods ✓ More than 61% of the nPCR positive cases were from Benishangul-Gumuz Region
4. Determine G6PD deficiency in endemic areas	I	✓ G6PD*A (A376G) (mild variant) was the only genotype detected, with national prevalence of 8.9% ✓ The severe G6PD deficiency allelic types, G6PD*A- (G202A) and Mediterranean (C563T), were not observed

8.1. Malaria sero-epidemiology

8.1.1. Characteristics of Study Participants

Of the expected 15,829 DBS samples, 10,278 individual DBSs were readily available for the current study the rest could not be found. Serological results were generated for 93.6% (9,622/10,278) of the available DBS (Figure 12). Six hundred fifty six samples failed or were insufficiently eluted from the filter paper. Serology data were generated for 9,622 samples of which 678 results were excluded for non-specific binding. Eight thousand nine hundred forty four results were merged with the primary data base (EMIS-2015) and included in the current report. One hundred ninety five results did not have demographic data and were excluded, as the barcode used failed to find a counter part in the database (Figure 12).

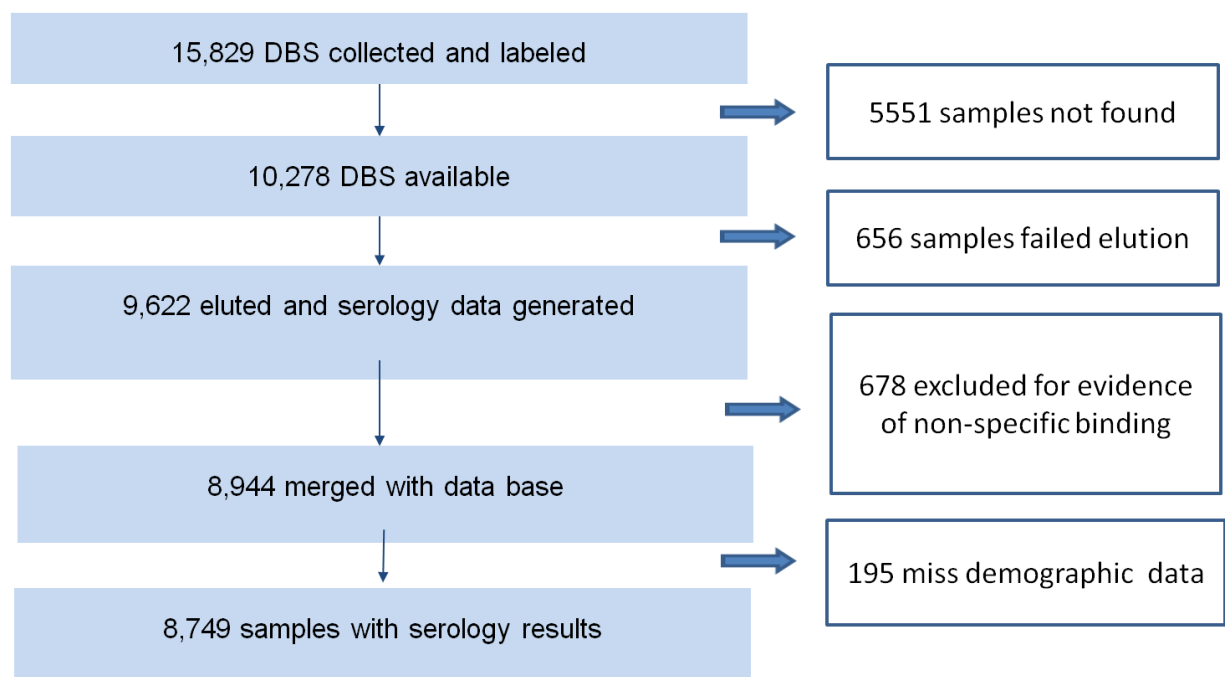


Figure 12. Flow chart showing sample selection and analysis procedure for serology study

Figure 13 shows the spatial distribution of sample collection households with serology data. Each point corresponds to up to 25 households in a selected EA. The number of individuals with serology data within an EA ranged from 1 to 55, with mean 24 individuals per EA. Areas above 2500 masl were excluded and regional sample allocation was proportional to population size.

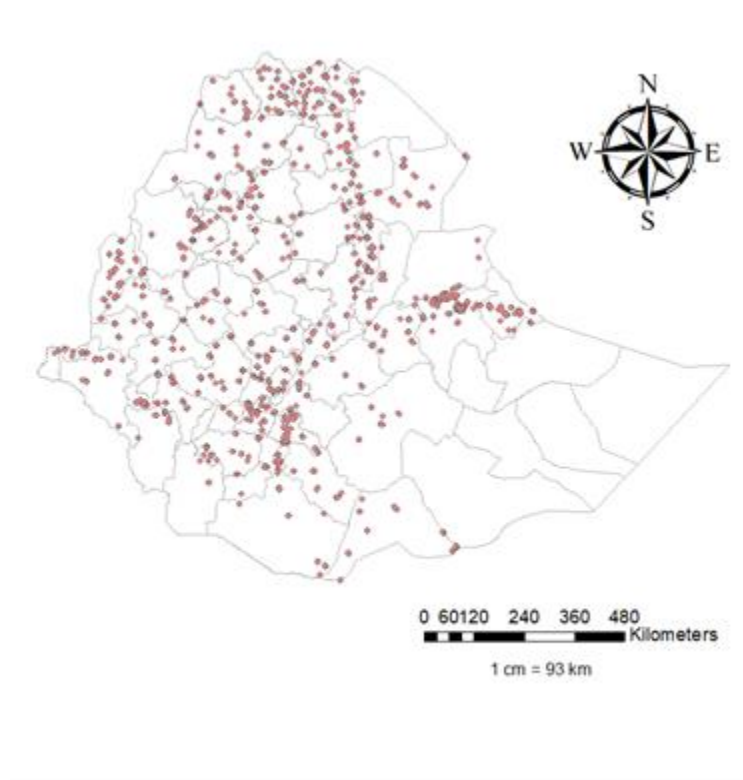


Figure 13. Distribution of Dried Blood Spot (DBS) samples collected in malaria endemic areas of Ethiopia, 2015. Each point depicts the sampled households in an enumeration area (N=8,749).

Characteristics of study participants by administrative region for persons providing blood sample and with serology data are presented in Table 4. Overall, females constituted 52.3% (95% CI: 51.1-55.9) of the survey participants. Mean age was 15.8 (95% CI: 15.2-16.4) (range: 1-107). The proportion of children under five years were 44.8% (95% CI: 43.0-46.5). Topographic heterogeneity were observed on the elevation of the study areas. Of the samples collected, 75.0% (95% CI: 67.5-81.2) were from areas with elevation less than 2000 m and 73.1% (95 CI: 65.6-79.4) were from elevation between 1000 and 2000m. Bed net use was reported by 62.1% (95% CI: 57.5-66.6) of respondents in the previous night and 0.8% (95% CI: 0.6-1.2) of the participants were positive by RDT; 0.5% (95% CI: 0.3-.7) for *P. falciparum* (HRP2 positive), 0.2% (95% CI: 0.1-0.4) for either *P. vivax*/*P. malariae*/*P. ovale* (Pv/Pm/Po) (panLDH positive) and 0.1% (95% CI: 0.1-0.3) considered a mixed infection (HRP2 and/or panLDH positive).

Table 4. National and regional characteristics of study participants for persons with serology data, EMIS-2015.

		Tigray	Afar	Amhara	Oromia	Somali	Benishangul-Gumuz	Southern Nations and Nationalities Peoples'	Gambela	Harari	Dire Dawa	National
Sex	Male	46.5 (44.1-48.9)	47.6 (42.9-52.3)	50.9 (47.4-54.3)	48.2 (45.6-50.9)	45.1 (40.8-49.4)	45.3 (40.8-49.9)	46.4 (44.3-48.5)	41.4 (37.4-45.5)	47.4 (3.0-65.4)	40.5 (33.1-48.4)	n =8743 47.7 (46.3-49.2)
	Female	53.3 (51.1-55.9)	52.4 (47.7-57.1)	49.1 (45.7-52.6)	51.8 (49.1-54.4)	54.9 (50.6-59.2)	54.7 (50.1-59.2)	53.6 (51.5-55.7)	58.6 (54.5-62.6)	52.6 (34.6-70.0)	59.5 (51.6-66.9)	52.3 (50.8-53.7)
Age group	0-5	43.8 (39.6-48.0)	66.6 (63.7-69.3)	40.8 (37.1-44.7)	44.6 (41.5-47.9)	61.7 (58.8-64.6)	49.5 (41.5-57.5)	45.4 (43.2-47.7)	54.8 (50.8-58.8)	25.5 (12.9-44.2)	46.0 (35.3-57.1)	44.8 (43.0-46.5)
	5-15	17.7 (15.2-20.4)	12.8 (9.2-17.5)	20.5 (18.0-23.3)	18.8 (15.8-22.3)	14.6 (11.7-18.1)	16.0 (12.9-19.6)	20.8 (18.9-22.9)	18.9 (16.6-21.5)	3.5 (1.0-11.6)	10.2 (6.6-15.4)	19.4 (17.8-21.1)
	15-25	13.3 (11.3-15.6)	6.1 (3.9-9.4)	12.6 (9.5-16.6)	11.6 (10.0-13.4)	5.2 (3.6-7.5)	11.5 (7.8-16.6)	11.4 (9.7-13.4)	10.7 (8.3-13.8)	22.1 (17.4-27.5)	14.0 (10.9-17.9)	11.7 (10.7-12.8)
	25-50	17.1 (14.9-19.4)	14.1 (12.1-16.4)	19.2 (16.2-22.6)	19.7 (17.5-22.1)	14.6 (13.0-16.4)	14.5 (10.7-19.3)	17.1 (15.6-18.6)	12.3 (1.1-15.0)	36.8 (29.3-45.0)	21.7 (15.9-28.9)	18.5 (17.3-19.8)
	50+	8.2 (6.6-10.1)	0.5 (0.1-1.5)	6.9 (5.1-9.1)	5.2 (3.9-7.0)	3.8 (2.7-5.3)	8.6 (5.3-13.5)	5.2 (4.1-6.6)	3.2 (2.3-4.5)	12.1 (7.4-19.2)	8.1 (4.5-14.2)	5.7 (4.9-6.6)
Elevation (m)	<1000	5.6 (1.6-17.4)	38.0 (13.7-70.4)	0	0	28.6 (12.6-52.7)	8.2 (1.1-52.7)	0	88.4 (72.1-95.8)	0	0.9 (0.1-6.7)	n=8740* 1.9 (1.2-2.8)
	1000-2000	64.3 (46.8-78.6)	62.0 (29.6-86.3)	53.4 (33.2-72.5)	77.4 (47.3-87.4)	71.4 (47.3-87.4)	91.8 (57.4-98.9)	79.0 (67.387.3)	11.6 (4.2-27.9)	100	99.1 (93.-99.9)	73.1 (65.6-79.4)
	>2000	30.2 (16.5-48.5)	0	46.6 (27.5-66.8)	22.6 (13.1-36.1)	0	0	21 (12.7-32.7)	0	0	0	25.0 (18.8-32.5)
Bed net use (last night)	Yes	57.6 (50.3-64.5)	80.5 (65.7-89.9)	59.1 (48.1-69.4)	69.6 (59.6-78.1)	75.2 (61.2-85.4)	66.1 (54.3-76.2)	55.7 (49.4-61.8)	84.7 (76.0-90.6)	33.5 (12.8-63.3)	25 (14.3-41.6)	n=5168** 62.1 (57.5-66.6)
	No	42.4 (35.5-49.7)	19.5 (10.1-34.3)	40.9 (30.6-51.9)	30.4 (21.9-40.4)	24.8 (14.6-38.8)	33.9 (23.845.7)	44.3 (38.2-50.6)	15.3 (9.4-24.0)	66.5 (36.7-87.2)	74.4 (58.4-85.7)	37.9 (33.4-42.5)
RDT* results	Pf	0.8 (0.4-1.6)	0	0.7 (0.2-1.8)	0.2 (0.1-0.6)	0	6.2 (2.2-16.3)	0.2 (0.1-0.5)	16.8 (11.8-23.2)	0	0	n=8732*** 0.5 (0.3-0.7)
	Pan	0.7 (0.3-1.3)	0.2 (0.0-1.3)	0	0.1 (0.0-0.4)	0.1 (0.0-0.8)	0	0.4 (0.1-1.2)	0.1 (0.0-0.6)	0	0	0.2 (0.1-0.4)
	Pf/ Pan	0.1 (0.0-0.6)	0	0.5 (0.1-1.8)	0	0	0.4 (0.0-2.6)	0.2 (0.0-0.6)	1.7 (0.9-3.0)	0	0	0.1 (0.1-0.3)
	Negative	98.4 (97.4-99.0)	99.8 (98.7-100.0)	98.9 (96.7-99.6)	99.7 (99.3-99.8)	99.9 (99.2-100.0)	93.5 (82.3-97.8)	99.3 (98.3-99.7)	81.5 (74.5-86.9)	100	100	99.2 (98.8-99.4)

n= number of observation, *RDT, Rapid Diagnostic Test, **three people did not have GPS coordinates, ***3613 individuals were without bed net use data and **** RDT test was not done for four individuals, four RDT tests were invalid and three individuals refused the RDT test.

By region, the proportion of females ranged from 49.1% (95% CI: 45.7-52.6) in Amhara to 59.5% (95% CI: 51.6-66.9) in Dire Dawa Region. The proportion of children under five years of age ranged from 25.5% (95% CI: 12.9-44.2) in Harari to 66.6% (95% CI: 63.7-69.3) in Afar Region; this proportion was above 60% in Afar and Somali regions. Above 73% of samples were collected between an elevation of 1000 and 2000 masl of Regions; this proportion was lowest in Gambela at 11.6% (95% CI: 4.2-27.9) and 100% in Harari Region. There was no sample collected for Amhara, Oromia, Southern Nations and Nationalities Peoples', and Harari Region from elevation below 1000m and there was no samples collected for Afar, Somali, Benishangul Gumuz, Gambela, Harari and Dire Dawa from an elevations above 2000 m. The highest bed net use in the previous night were observed in Gambela 84.7% (95% CI: 76.0-90.6) and Afar regions 80.5% (95% CI: 65.7-89.9), the lowest in Dire Dawa Region 25% (95% CI: 14.3-41.6). More than 90% of the positive *P. falciparum* RDT results were from Gambela 16.8% (95% CI: 11.8-23.2) and Benishangul-Gumuz 6.2% (95% CI: 2.2-16.3). The highest non *P. falciparum* (pan) cases were reported in Tigray Region 0.7% (95% CI 0.3-1.3). There were no positive RDT cases in Harari and Dire Dawa regions (Table 4).

8.1.2. National seroprevalence

Table 5. Proportion of individuals (all ages and under five) malaria seropositive by species and by antigen in malaria endemic areas of Ethiopia, 2015. An individual was considered positive if they had a positive antibody response either for Merozoite Surface Protein-1 (MSP-1) or Apical Membrane Antigen-1 (AMA-1) antigens for each species.

<i>Plasmodium</i> species	Antigens	Percent seropositive (95% CI) (All ages)	Percent Seropositive (95% CI) (Under five)
<i>P. falciparum</i>	MSP-1 AMA-1 Pf positive (MSP-1 and/or AMA-1)	23.8 (21.4-26.3) 24.5 (22.4-26.7) 32.1 (29.8-34.4)	12.3 (10.6-14.2) 11.3 (9.8-13.0) 18.0 (16.1-20.1)
<i>P. vivax</i>	MSP-1 AMA-1 Pv positive (MSP-1 and/or AMA-1)	21.2 (19.2-23.4) 14.4 (12.7-16.2) 25.0 (22.7-27.3)	12.3 (10.5-14.4) 6.7 (5.5-8.0) 14.8 (12.7-17.2)
<i>P. malariae</i> ¹	MSP-1	8.6 (7.6-9.7)	6.1 (5.0-7.3)
<i>P. ovale</i> ¹	MSP-1	3.1 (2.5-3.8)	2.0 (1.4-2.7)
Any <i>plasmodium</i>	Pf (MSP-1/AMA-1) or Pv (MSP-1/AMA-1) or Pm MSP-1 or Po MSP-1	44.5 (41.9-47.2)	30.2 (27.3-33.2)
Either <i>P. falciparum</i> or <i>P. vivax</i>	Pf (MSP-1/AMA-1) or Pv (MSP-1/AMA-1)	41.1 (38.5-43.7)	26.7 (24.0-29.5)
Both <i>P. falciparum</i> and <i>P. vivax</i>	Pf (MSP-1/AMA-1) and Pv (MSP-1/AMA-1)	6.6 (5.5-8.0)	1.7 (1.2-2.4)

¹Normal approach (mean seronegative +3sd) to defining cut-off used for *P. malariae* and *P. ovale*

Proportion of malaria seropositive individuals by species and by antigen in malaria endemic areas of Ethiopia are presented in Table 5. Nationally, 32.1% (95% CI: 29.8-34.4) of the participants were seropositive for any IgG against the two *P. falciparum* antigens (MSP-1/AMA-1), and 25.0% (95% CI: 22.7-27.34) for *P. vivax*. In the current study, 8.62% (95% CI: 7.6-9.7) and 3.1% (95% CI: 2.5-3.8) seropositivity were observed for *P. malariae* and *P. ovale*, respectively; this made the total proportion of seropositive samples for any *plasmodium* species 44.5% (95% CI: 41.9-47.2). Forty one point one percent (95% CI: 38.5-43.7) of individuals were positive either for *P.*

falciparum or *P. vivax*, with 6.6% (95% CI: 5.5-8.0) of participants seropositive for both *P. falciparum* and *P. vivax*. By antigen, 23.8% (95% CI: 21.4-26.3) were seropositive against *P. falciparum* MSP-1 and 24.5% (95% CI: 22.4-26.7) AMA-1, while for *P. vivax*, 21.2% (95% CI: 19.2-23.4) were seropositive against MSP-1 and 14.4% (95% CI: 12.7-16.2) AMA-1 (Table 5).

The prevalence proportions for children under five were lower compared to all age groups. Seropositive for *P. falciparum* were 18.0% (95% CI: 16.1-20.1) and 14.8 % (95% CI: 12.7-17.2) for *P. vivax*. Similarly, 6.1% (95% CI: 5.0-7.3) and 2.0% (95% CI: 1.4-2.7) children under five were seropositive for *P. malariae* and *P. ovale*, respectively (Table 5). The total proportion of children positive for any *Plasmodium* species were 30.2% (95% CI: 27.3-33.2) and 26.7% (95% CI: 24.0-29.5) were seropositive either for *P. falciparum* or *P. vivax*; 1.7% (95% CI: 1.2-2.4) were seropositive both for *P. falciparum* and *P. vivax*. By antigen, 12.3 % (95% CI: 10.6-14.2) under five children were seropositive against *P. falciparum* MSP-1 and 11.3% (95% CI: 9.8-13.0) AMA-1, and for *P. vivax*, 12.3% (95% CI: 10.5-14.4) were seropositive against MSP-1 and 6.7% (95% CI: 5.5-8.0) AMA-1.

Males were slightly more exposed to malaria infection compared to females, nationally 33.3% (95% CI: 30.4-36.2) of the males were seropositive for *P. falciparum* compared to 30.9% (95% CI: 28.3-33.6) of the females [aOR: 1.25 (95% CI: 1.1-1.4), $p < 0.05$]. The trend was similar for *P. vivax* while with a non-significant p-value, where 26.4 (95% CI: 23.4-29.7) of males were seropositive compared to 23.6% (95% CI: 21.1-26.3) of females [aOR: 1.27 (95% CI: 1.08-1.49), $p > 0.05$] (Table 6).

Table 6. Percent malaria seropositivity and Odds Ratio (OR) by sex, age and elevation for each species in malaria endemic areas of Ethiopia,2015.

	<i>P. falciparum</i>			<i>P. vivax</i>		
	% Seropositive (MSP-1 or AMA-1)	Unadjusted OR (95% CI)	Adjusted OR (95% CI)‡	% Seropositive (MSP-1 or AMA-1)	Unadjusted OR (95% CI)	Adjusted OR (95% CI)‡
Sex						
Female	30.9 (28.3-33.6)	Ref	Ref*	23.6 (21.1-26.3)	Ref	Ref
Male	33.3 (30.4-36.2)	1.11 (0.99- 1.23)	1.25 (1.10-1.42)	26.4 (23.4-29.7)	1.16 (0.99- 1.37)	1.27 (1.08-1.49)
Age (Years)						
<5	18.0 (16.0-20.2)	Ref**	Ref**	14.8 (12.7-17.3)	Ref**	Ref**
5-15	31.5 (27.6-35.6)	2.18 (1.9-2.5)	2.26(1.98-2.58)	25.7 (22.5-29.2)	2.52(2.19-2.90)	2.54 (2.17-2.96)
15-25	43.7 (38.6-48.8)	3.82 (3.3-4.5)	4.04(3.45-4.72)	34.0 (28.7-39.8)	4.64 (3.95-5.46)	4.52 (3.81-5.37)
25-50	50.5 (45.4-55.5)	5.2 (4.6-5.9)	6.50(5.63-7.49)	36.7 (32.7-40.8)	6.57 (5.71-7.57)	4.87 (4.20-5.65)
≥50	58.2 (52.0-64.2)	6.32 (5.1-7.8)	7.99(6.42-9.93)	43.7 (36.8-50.8)	8.30 (6.69-10.30)	6.16 (4.97-7.64)
Elevation (meters)						
>2000	24.4 (20.6-28.6)	Ref**	Ref**	20.9 (17.4-24.9)	Ref	Ref
1000-2000	34.3 (31.4-37.3)	1.6 (1.26-2.07)	1.83 (1.40-2.41)	26.4 (23.4-29.5)	1.35 (1.03-1.78)	1.46 (1.10-1.95)*
<1000	49.9 (40.3-59.5)	3.08 (1.98-4.81)	4.36 (2.71-7.03)	24.6 (17.2-33.9)	1.23 (0.74-2.05)	1.49 (0.94-2.34)

*P-values <0.05; ** P-values <0.01‡ Controlled for listed risk factors

8.1.3. Seroprevalence by age

The DBS sample collection was purposely biased towards children under five years of age, with 43.8 % (95% CI: 39.6-48.0) of the DBS collected from this age group (Table 3). Figure 14 shows the national seropositivity by age groups for *P. falciparum* and *P. vivax*. The comparison of five age groups showed there was a significant increase in seropositivity with increasing age group ($p < 0.01$). The highest seropositivity was observed in the oldest age group >50 years (Figure 14). Adults are at least six times more likely exposed to malaria infection compared to under five children. The adjusted odds ratio for *P. falciparum* was [aOR: 7.99 (95% CI: 6.42-9.93), $P < 0.01$] and for *P. vivax* was [aOR: 6.16 (95% CI: 4.97-7.64), $P < 0.01$] (Table 6). The pattern was more pronounced for *P. falciparum* compared to *P. vivax*, although the difference is highly significant for both species ($p < 0.01$) (Figure 14)

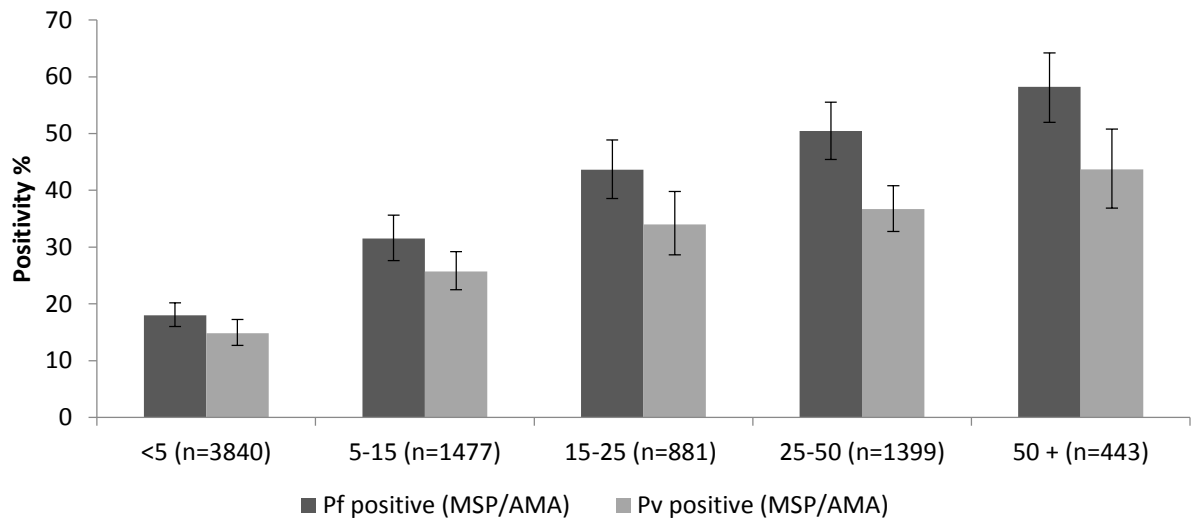


Figure 14. National seropositivity by age group for *P. falciparum* and *P. vivax*, 2015. An individual was considered positive if they had a positive antibody response either for Merozoite Surface Protein-1 (MSP-1) or Apical Membrane Antigen-1 (AMA-1) antigens for each species. n was the number of samples tested.

8.1.4. Seroprevalence by elevation

Seropositivity for *P. falciparum* and *P. vivax* by five elevation stratification (<500, 500-1000, 1000-1500, 1500-2000 and >2000 masl) for all age (A) and under five children (B) are presented in Figure 15. Variability was observed on the prevalence of seropositivity over each strata by species. Seropositivity was inversely related to elevation for *P. falciparum*, with lower elevation (<2000 masl) areas having significantly higher seroprevalence compared to higher elevations (2000-2500 masl) [aOR 4.36 (95% CI: 2.7 -7.0), $p<0.01$] (Table 6). The highest seropositivity for *P. falciparum* was observed at elevations below 1500 masl and a peak was observed at an elevation below 1000 masl (Figure 15.A). *P. vivax* seropositivity increased with increasing elevation from less than 500 to 1000 masl. The highest *P. vivax* seropositivity was observed at an elevation of 1500-2000 masl and seropositivity slightly decreases above 2000 masl (Figure 15.A). However, a weaker inverse effect of elevation was observed for *P. vivax*. There was no significant difference in the transmission of *P. vivax* on elevation strata above and below 2000 masl [aOR 1.5 (95% CI: 0.9-2.4), $p>0.05$] (Table 6).

Among children under five years of age, *P. falciparum* and *P. vivax* show differing relationship with elevation. *P. falciparum* seropositivity increased with increasing elevation, the highest *P. falciparum* seropositivity was observed below 1000 masl [aOR 3.1 (95% CI: 1.3 -3.6), $p<0.01$] (Figure 15.B, Table 6). Seropositivity for children under five years of age showed a direct relationship for *P. vivax* and elevation (Figure 15.B). The highest seropositivity was observed between 1000 and 2000 masl m [AOR 1.5 (95% CI: 1.1 -2.05), $p<0.05$] (Table 6). The elevation variability for *P. malariae* and *P. ovale* seropositivity was minimal, a slight increase in the seropositivity of both species was observed in lower elevations (less than 2000 m) compared to higher elevation, with non-significant statistics ($P<0.05$).

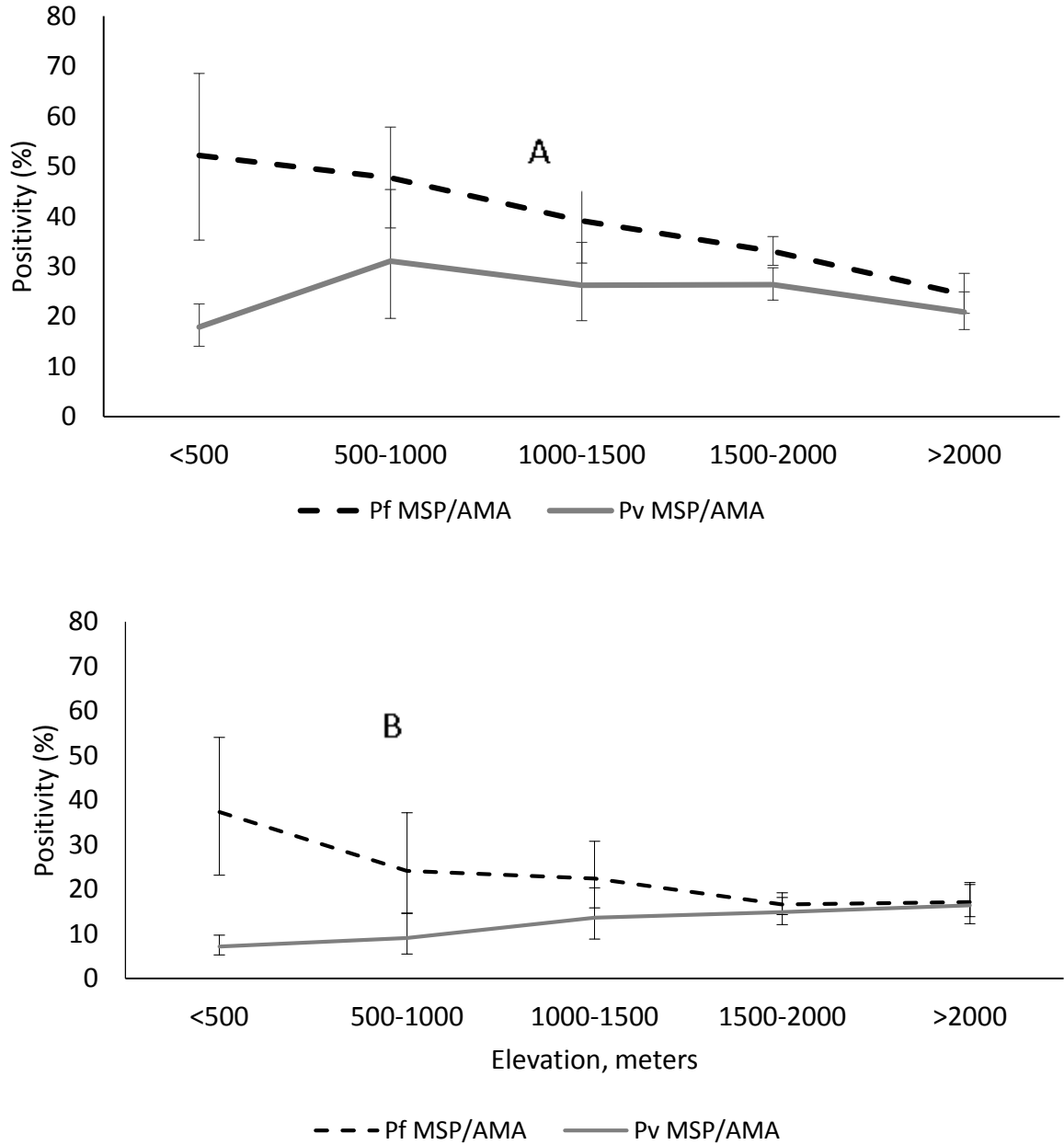


Figure 15. Seropositivity by five elevation categories (< 500, 500-1000, 1000-1500, 1500-2000 and > 2000 masl) for *P. falciparum* Merozoite Surface Protein-1 (MSP-1) or Apical Membrane Antigen-1 (AMA-1), and *P. vivax* MSP-1 or AMA-1, 2015. An individual was considered positive if they had a positive antibody response either for MSP-1 or AMA-1 antigens for each species. Broken line depicts *P. falciparum* (MSP-1/AMA-1) and solid line represent *P. vivax* (MSP-1/AMA-1). Error bars indicate 95% confidence interval A. All ages. B. Children under five years of age.

8.1.5. Seroprevalence by region

The proportion of individuals seropositive by region is shown in Figure 16 A and B. Figure 16 A shows seropositivity across the regions, with *P. falciparum* seropositivity higher than *P. vivax* seropositivity in all the regions. The proportion seropositive *P. falciparum* by region ranged from 11.0% (95% CI: 8.8-13.7) in Somali to 65.0% (95% CI: 58.0-71.4) in Gambela Regions. For *P. vivax*, seropositivity ranged from 4.0% (95% CI: 2.6-6.2) in Somali to 36.7% (95% CI: 30.0-44.1) in Amhara Regions (Figure 16 A). The trend of seropositivity was similar in under five children with small magnitude of proportion in under five children (Figure 16 B).

For the rare species *P. malariae* and *P. ovale* seropositivity, variability was observed over the regions. *P. malariae* seropositivity ranged from zero to eight percent; above five percent proportion was reported for Gambela, Oromia and Amhara regions. *P. ovale* seropositivity ranged from zero to five percent; above one percent seroprevalence for *P. ovale* was reported in SNNP, Benishangul Gumuz, and Amhara and Oromia regions. A conservative estimation for the rare species is reported in section 8.1.6.

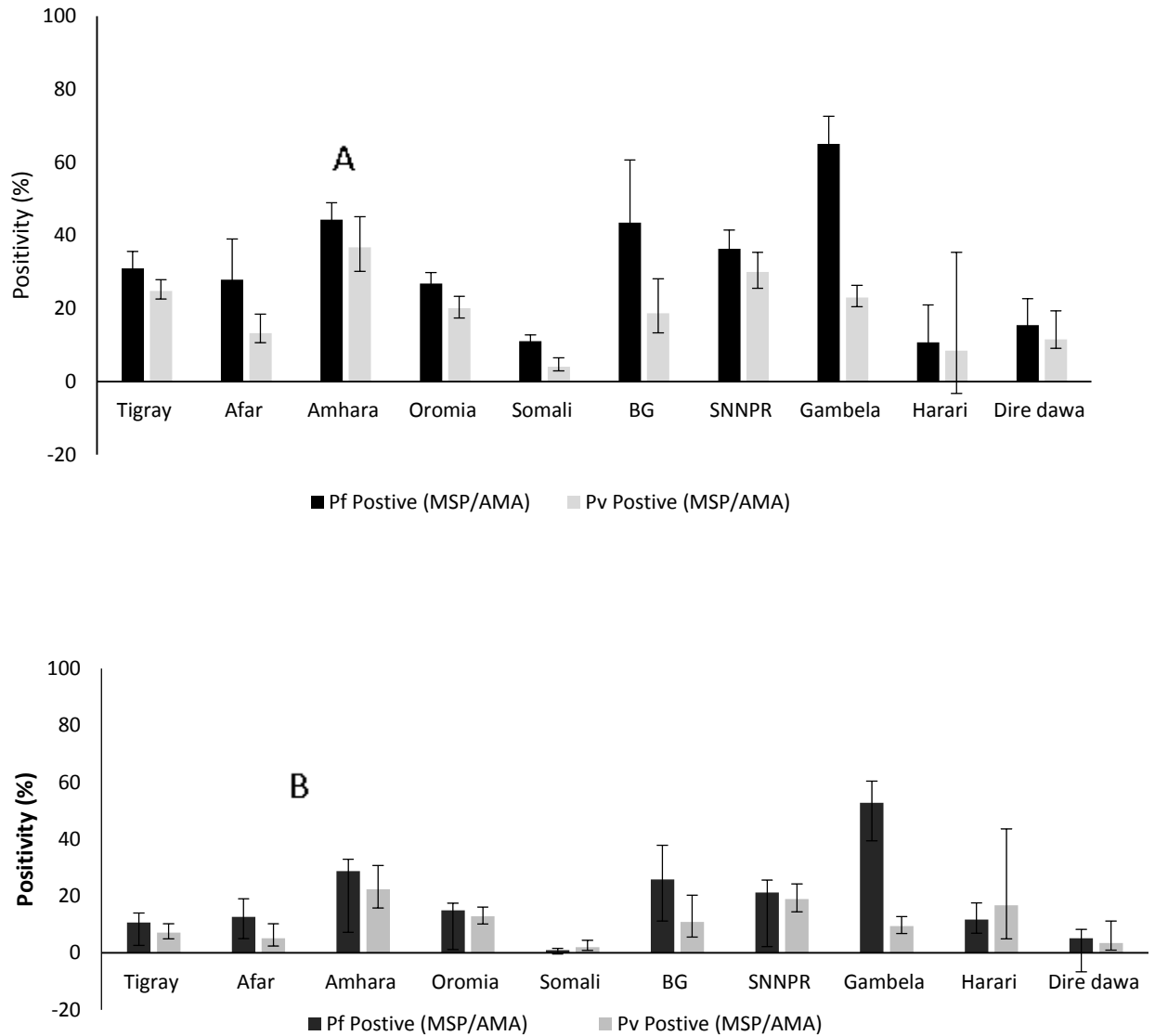


Figure 16. Seropositivity by region for *P. falciparum* and *P. vivax*. Individuals were considered positive, if they had a positive antibody response either for Merozoite Surface Protein-1 (MSP-1) or Apical membrane Antigen-1 (AMA-1) antigens for each species, 2015. Error bars indicate 95% confidence interval. (BG: Benishangul Gumuz and SNNPR: Southern Nations and Nationalities People's Region). A. All ages B. Children under five years of age.

8.1.6. Conservative estimate for *P. malariae* and *P. ovale*

Table 7 shows proportion of seropositive individuals for *P. malariae* and *P. ovale* by region based on the conservative approach to defining seropositive cut-off values. Of all samples tested, 5.1% (95% CI: 4.4-6.0) were seropositive for *P. malariae* and 1.5% (95% CI: 1.1-2.0) for *P. ovale*. Similarly, 4.6% (95% CI: 3.6-5.7) and 1.2% (95% CI: 0.8-1.9) of under five children were seropositive for *P. malariae* and *P. ovale*, respectively. Both species were observed in all the regions, albeit with a variable distribution. Seropositivity ranged from 0.2% (95% CI: 0.1-1.1) in Gambela to 6.3% (95% CI: 5.0-7.8) in Oromia Regions for *P. malariae* and 0.1 % (95% CI: 0.0-0.5) in Somali to 4.0% (95% CI: 0.3-33.6) in Harari Regions (note: large confidence interval). A similar trend of seropositivity was observed for under five children, however, zero seropositivity was reported for *P. ovale* in Somali, Gambela, Harari and Dire Dawa Regions for under five children (Table 7).

Table 7. Proportion seropositive for *P. malariae* [Merozoite Surface Protein-1 (MSP -1)] and *P. ovale* (MSP-1), using the conservative approach to defining seropositive cut-off values, 2015.

Region	All ages		Under five	
	Pm-MSP-1	Po-MSP-1	Pm-MSP-1	Po-MSP-1
Tigray	1.1 (0.6-2.3)	0.6 (0.3-1.1)	1.1 (0.4-2.9)	0.3 (0.1-1.2)
Afar	1.3 (0.6-3.0)	0.6 (0.1-2.7)	1.8 (0.7-4.2)	0.5 (0.1-4.2)
Amhara	5.0 (3.2-7.6)	2.4 (1.8-3.3)	2.7 (1.1-6.2)	1.0 (0.3-3.1)
Oromia	6.3 (5.0-7.8)	2.0 (1.3-3.0)	5.8 (4.4-7.8)	1.8 (1.0-3.3)
Somali	1.3 (0.6-2.8)	0.1 (0.0-0.8)	1.1 (0.4-2.9)	0
Benishangul-Gumuz	2.1 (1.0-4.4)	0.7 (0.2-2.5)	1.3 (0.4-4.7)	1.5 (0.4-4.9)
SNNP	4.9 (3.7-6.3)	0.6 (0.4-1.2)	4.7 (3.1-7.1)	0.7 (0.3-1.8)
Gambela	0.2 (0.1-1.1)	0.1 (0.0-0.5)	0.3 (0.1-1.2)	0
Harari	4.5 (0.6-26.4)	4.0 (0.3-33.6)	0	0
Dire Dawa	2.4 (0.7-8.0)	0.8 (0.1-5.3)	1.7 (0.3-9.9)	0
Total (%)	5.1 (4.4-6.0)	1.5 (1.1-2.0)	4.6 (3.6-5.7)	1.2 (0.8-1.9)

8.1.7. Seroconversion rates

The age seroconversion plots for antibody responses for both *P. falciparum* and *P. vivax* are shown in Figures 17-19. Seroconversion plots/curves represent the rate at which a population become seropositive to specific antigens resulting in seroconversion rates (SCR) or lambda (λ). The seropositivity responses were fitted to a model that predicted the maximum likelihood seropositivity over the ages, with the 95% confidence interval and SCR, per year. The model also predicted the probability of seropositivity by age that fits with the observed data.

The national age-seroconversion plots for antibody responses for *P. falciparum* and *P. vivax* antigens are shown in Figure 17. In general, a higher seroconversion rates were predicted for *P. falciparum* compared to *P. vivax* for the combined MSP-1/AMA-1 antigens: national SCR per year for *P. falciparum* was 0.084 (95% CI: 0.076-0.091) and for *P. vivax* 0.049 (95% CI: 0.045-0.055). Both the individual MSP-1 and AMA-1 antigens also show similar trend where there was high SCR for *P. falciparum* compared to *P. vivax*. For *P. falciparum*: MSP-1 0.06 (95% CI: 0.063-0.076) and AMA-1 0.049 (95% CI: 0.045-0.054) compared to *P. vivax*: MSP-1 0.042 (95% CI: 0.037-0.047) and AMA-1 0.022 (95% CI: 0.020-0.025). A similar trend was observed when the model was fitted to data with an elevation of below 2000 masl both for the combined and individual antigens. Generally, MSP-1 show higher seroconversion rates compared to AMA-1 antigens for both species and heterogeneity was observed across the different antigen responses.

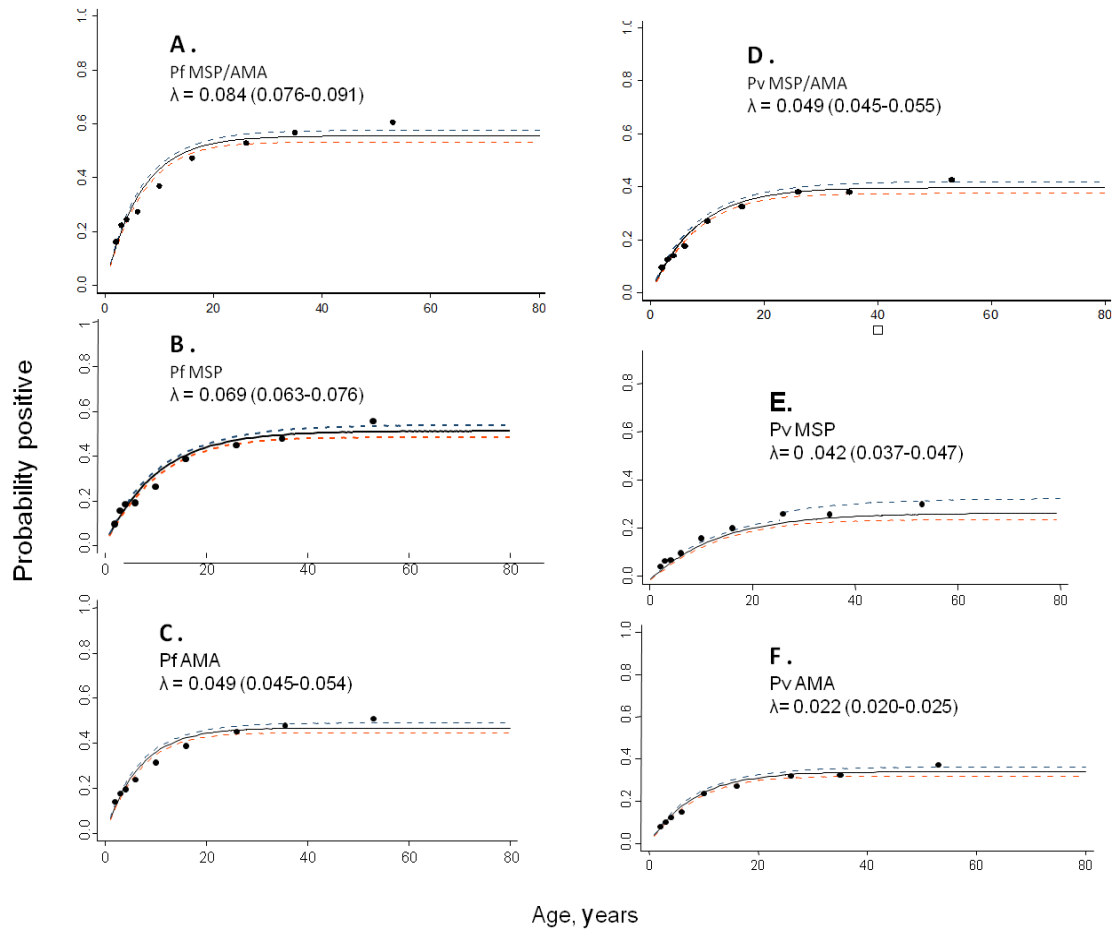


Figure 17. National age-seroconversion plots for antibody responses for *P. falciparum* and *P. vivax* antigens (MSP-1, AMA-1), 2015. Note: Y axis represent probability seropositive over age. Seroconversion curves represent the rate at which a population become seropositive to specific antigens resulting in seroconversion rates (SCR) or lambda (λ). In each graph points represent age seroprevalence (by deciles), unbroken line represent maximum likelihood curves and broken lines represent the 95% confidence interval. Plots A and B depict the seroconversion rates for combined (MSP-1/AMA-1) *P. falciparum* antigen response (A) and for combined (MSP-1/AMA-1) *P. vivax* antigen response (D). Plots B and C show seroconversion rates for individual *P. falciparum* antigens MSP-1 (B) and AMA-1(C).Plots E and F show seroconversion rates for individual *P. vivax* antigens MSP-1 (E) and AMA-1(F).

The log likelihood model was fitted to data generated from the four major administrative regions (Amhara, Oromia, SNNP and Tigray) for *P. falciparum* and *P. vivax* MSP-1 and AMA-1 antigen

responses, separately. Heterogeneous seroconversion rates were predicted over the regions for *P. falciparum* and *P. vivax* (Figure 18 and 19). Variability was also observed on the response of the MSP-1 and AMA-1 antigens within the same region, overall higher SCR was reported to MSP-1 antigen responses compared to AMA-1 (Figures 18 and 19).

Figure 18 show the SCR for *P. falciparum* MSP-1 and AMA-1 antigen responses. SCR ranges from 0.033 (95% CI: 0.025-0.044) in Oromia to 0.110 (95% CI: 0.084-0.143) in Amhara by MSP-1 and 0.031 (95% CI: 0.025-0.039) in Oromia to 0.064 (95% CI: 0.051-0.079) in SNNP region by AMA-1 antigen. The SCR in Amhara was still higher at 0.060 (95% CI: 0.051-0.079).

For *P. vivax* seroconversion rates range from 0.027 (95% CI: 0.017-0.043) in Tigray to 0.082 (95% CI: 0.062-0.107) in Amhara by MSP-1 and from 0.017 (95% CI: 0.013-0.022) in Tigray to 0.042 (95% CI: 0.032-0.0569) in SNNP region by AMA-1 antigen Figure 19.

AMA-1 antigen showed a relatively high SCR in SNNP Region for both *P. falciparum* and *P. vivax* responses. MSP-1 showed higher SCR for all the regions compared to AMA-1 except for SNNP Region (Figure 18 and 19). Generally, among the four regions SCR was higher in Amhara and lower in Tigray Regions.

For seroconversion plots, where visual inspection indicated better fit between the observed and predicted pattern of transmission, two forces of infection were compared with a single force of infection. The visual Inspection was guided by profile likelihood plots. The data fitted to two forces of infection depict a change in malaria transmission over the last 15 years both *P. falciparum* and *P. vivax* antigen responses, however the changes observed were not statistically significant ($P > 0.05$) (Annex 1). There was a considerable variation on the fitting of the model between the antigens and among the regions.

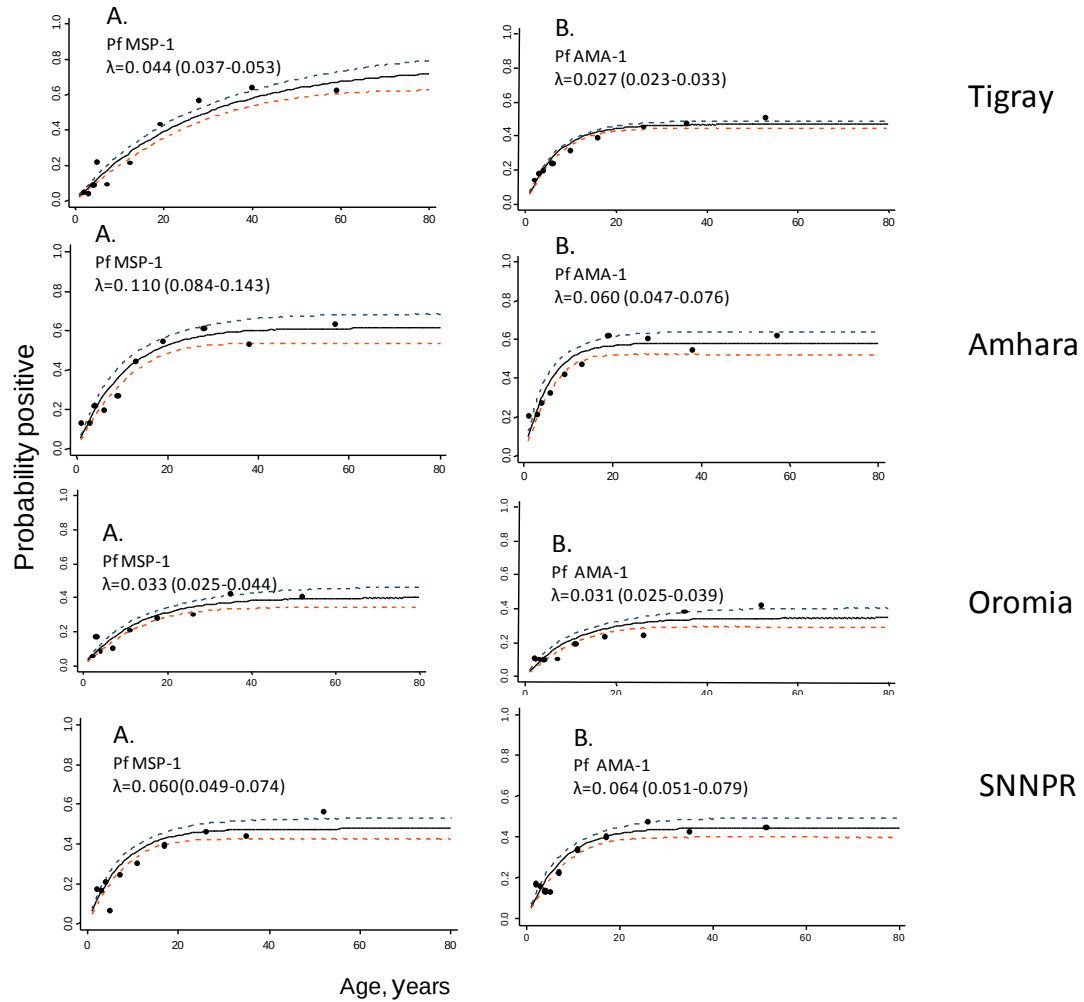


Figure 18. Regional age-seroconversion plots for antibody responses for *P. falciparum* antigens, 2015. Note: Horizontal lines represent probability seropositive over age. Seroconversion curves represent the rate at which a population becomes seropositive to specific antigens resulting in seroconversion rates (SCR) or lambda (λ). Each graph point represents age seroprevalence (by deciles), unbroken line represents maximum likelihood curves and broken lines represent the 95% confidence interval. Plots A and B depict the seroconversion curves for *P. falciparum* antigens response to MSP-1 (A) and AMA-1(B), for the major four regions Tigray, Amhara, Oromia and Southern Nations and Nationalities People's Regions (SNNPR).

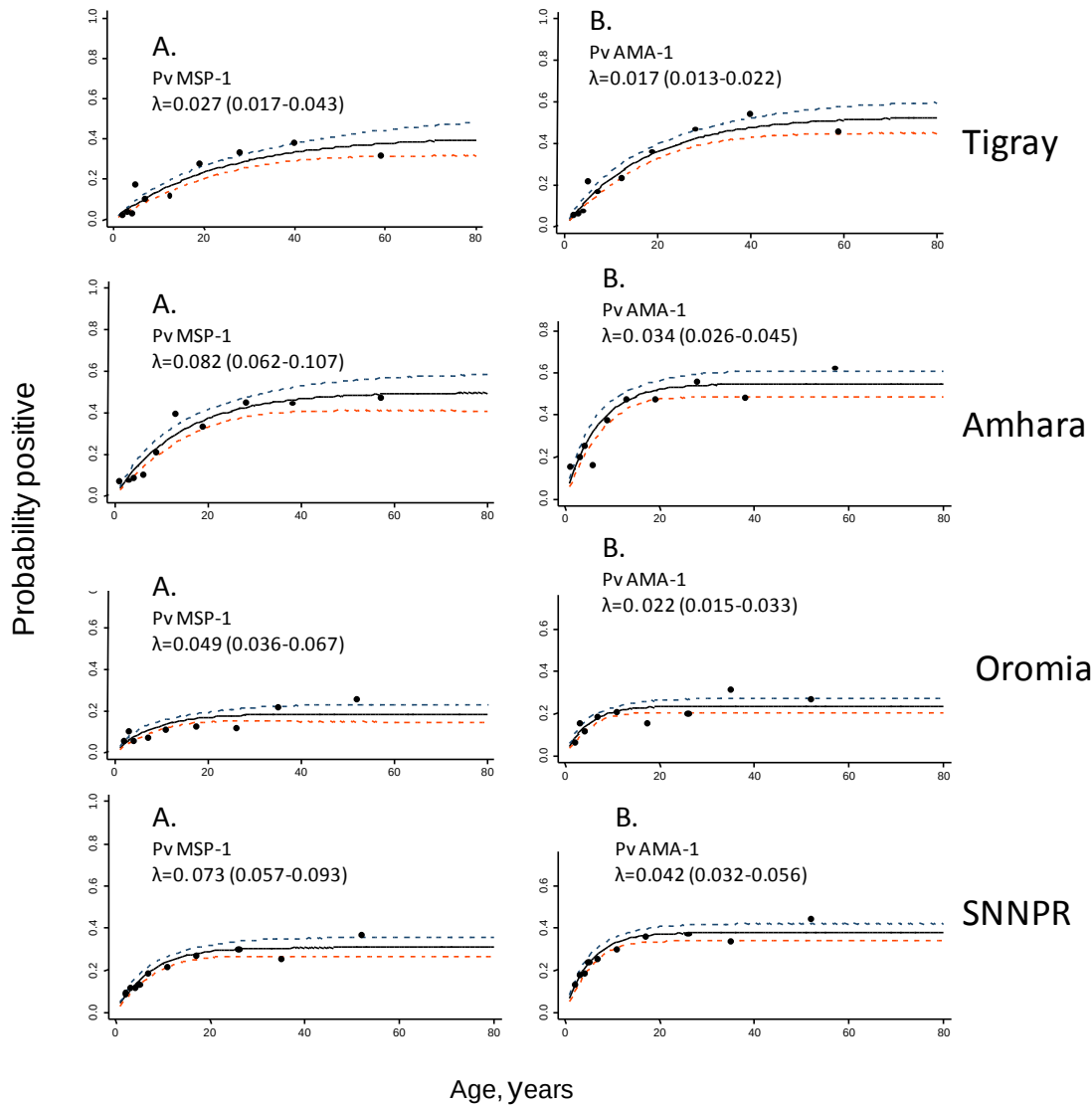


Figure 19. Regional age-seroconversion plots for antibody responses for *P. vivax* antigens, 2015. Horizontal lines represent probability seropositive over age. Seroconversion curves represent the rate at which a population become seropositive to specific antigens resulting in seroconversion rates (SCR) or lambda (λ). Each graph point represents age seroprevalence (by deciles), unbroken line represents maximum likelihood curves and broken lines represent the 95% confidence interval. Plots A and B depict the seroconversion curves for *P. vivax* antigen response to MSP-1 (A) and AMA-1 (B), for the major four regions Tigray, Amhara, Oromia and Southern Nations and Nationalities People's Region (SNNPR).

8.1.8. Spatial distribution of seroprevalence

An interpolated surface map for *P. falciparum* and *P. vivax* antigen responses are depicted in Annex 3. Generally, malaria transmission is predicted to be higher in the North West compared to East of the country. The spatial pattern of transmission was predicted to be similar for both species with higher intensity for *P. falciparum* MSP-1 and AMA-1 antigen responses (Annex 3: Pf AMA and Pf MSP) compared to *P. vivax* MSP-1 and AMA-1 antigen responses (Annex 3: Pv AMA and Pv MSP).

Similarly, an interpolated surface maps for *P. falciparum* and *P. vivax* were produced using data from the Annual Parasite Index 2014 (API 2014). Between the two antigens, *P. falciparum* MSP-1 showed high correlation with API 2014 (spearman correlation 0.37, $P < 0.01$) and *P. vivax* AMA-1 show better correlation with API 2014 *P. vivax* prevalence data (spearman correlation 0.32, $P < 0.01$) (Annex 4). Visual inspection show similarity between predicted positive probability surface correspond to malaria risk as noted from the API in 2014 (Annex 4).

In a separate map (Annex 2), spatial clustering of seroprevalence for *P. falciparum* and *P. vivax* was depicted. Maps were produced for adults and children under five years of age for the combined MSP-1 and AMA-1 responses for both species. Higher seropositivity and clustering was observed for all age groups compared to children under five years of age for both species.

Generally, seropositivity was found to be variable in space, across elevation strata and regions. An interpolated surface map predicted differing transmission pattern for *P. falciparum* and *P. vivax*.

8.2. Subpatent infection

8.2.1. Study population

Of 2,669 samples available from three regions 2,535 samples were pooled for PCR analysis, 945 Amhara, 668 Benishangul Gumuz and 922 Tigray Regions (Figure 11). A total of 2,608 samples had complete assay and demographic data (2533 RDT negative, 73 RDT positive and 8 had no RDT results). About 53% (95% CI: (50.4-54.2)) of the survey participants were female and 44% (95% CI: 42.0-45.8) of sample were collected from children under five years of age (Table 8). Mean age was 16.1 years (95% CI: 15.4-17.0) (range: 1-88 years). About 80% of the sample were collected from areas with an elevation of less than 2000 m. A total of 41.3% (95% CI: 38.7-43.9) of the participants were seropositive for *P. falciparum* MSP-1 or AMA-1 antigen responses and 34.0% (95% CI: 31.6-36.6) were seropositives for *P. vivax*. Additionally, 7.0% (95% CI: 5.7-8.4) and 4.9 % (95% CI: 3.8-6.2) of participants were seropositive for *P. malariae* and *P. ovale* MSP-1 antigen responses, respectively. 59.6% (95% CI: 57.3-61.9) of respondents reported having slept under a bed net in the previous night (Table 8).

Table 8. Characteristics of survey samples collected from northwestern Ethiopia for persons with nPCR data, 2015.

Characteristics	Number	Proportion (95% CI)
Sex	N=2,608	
Female	1,364	52.3 (50.4-54.2)
Male	1,244	47.7 (45.8-49.6)
Age	N=2,608	
<5	1,145	43.6 (42.0-45.8)
5-15years	508	19.5 (18.0-21.0)
15-25 years	325	12.5 (11.2-13.8)
25-50 years	460	17.6 (16.2-19.1)
≥50 years	170	6.5 (5.6-7.5)
Elevation		
<1000 m	144	5.5 (4.7- 6.5)
1000-2000 m	1,990	76.3 (74.6-77.9)
>2000 m	474	18.2 (16.7-19.7)
Use of bed nets	N=1,798	
Yes	1,072	59.6 (57.3-61.9)
No	726	40.4 (38.1-42.7)
Seropositivity (MSP-1/AMA-1)	N= 1,354	
<i>P. falciparum</i>	559	41.3 (38.7-43.9)
<i>P. vivax</i>	461	34.0 (31.6-36.6)
<i>P. malariae</i>	94	7.0 % (5.7-8.4)
<i>P. ovale</i>	66	4.9 % (3.8-6.2)

8.2.2. Prevalence of *Plasmodium spp* specios infection

The percent positivity of malaria by RDT, microscopy, PCR and serology diagnostic method by species is presented in Table 9. Of the 2,608 samples tested 2.8 % (95% CI: 2.2-3.5) were positive for malaria by RDTs; among them, 1.9% (95% CI: 1.5-2.5) were *P. falciparum*, 0.4% (95% CI: 0.2-0.7) were *P. falciparum* mixed with other species and 0.5% (95% CI: 0.3-0.5) were either *P. vivax*, *P. malariae* or *P. ovale*. By microscopy, 1.2% (95% CI: 0.8-1.7) were positive for malaria, of which 1.0 % (95% CI: 0.6-1.4) were *P. falciparum*, 0.2% (95% CI: 0.0-0.4) were *P. vivax* and

0.08% (95% CI: 0.0-0.3) were mixed *P. falciparum* and *P. vivax* infection. RDT positivity was over two-fold higher compared to microscopy.

Among the tested samples from the three regions, DNA screening by nPCR detected 3.3 % (95% CI: 2.7-4.1) positivity for any malaria species. *P. falciparum* accounted for 3.1% (95% CI: 2.5-3.8), *P. vivax* 0.4% (95% CI: 0.2-0.7), mixed (*P. falciparum* and *P. vivax*) 0.1% (95% CI: 0.0-0.4), mixed (*P. falciparum* and *P. malariae*) 0.1 % (95% CI: 0.0-0.3). PCR detected 64.4% and 16.1% more positives compared to microscopy and RDT, respectively ($P < 0.05$). In addition, PCR detected two *P. malariae* cases mixed with *P. falciparum* cases. By RDT, this were identified as *P. falciparum* or mixed (*P. vivax*, *P. ovale* or *P. malariae*) and as *P. falciparum* monoinfection. By microscopy one of the *P. malariae* was missed and the other was identified as mixed infection. No *P. ovale* was identified among the diagnosed samples by microscopy or nPCR diagnostic methods (Table 9).

Table 9. Percent malaria positivity of samples diagnosed by Rapid Diagnostic Test (RDT), Microscopy and nested Polymerase Chain Reaction (nPCR) and seropositivity for three regions (Tigray, Amhara and Benishangul Gumuz), EMIS 2015

Diagnostic methods	Species diagnosed	Number N=2608	Percent positivity (95% CI)	Percent difference from PCR
RDT	<i>P. falciparum</i>	50	1.9 (1.5-2.5)	37.5
	<i>P. vivax</i> / <i>P. malariae</i> / <i>P. ovale</i>	12	0.5 (0.3-0.8)	0.0
	<i>P. falciparum</i> mixed (Pv/Pm/Po)	11	0.4 (0.2-0.7)	-120.0
	Total <i>Plasmodium spp</i> positive n= 2,606*	73	2.8 (2.2-3.5)	16.1
Microscopy (slide positivity)	<i>P. falciparum</i>	25	1.0 (0.6-1.4)	68.8
	<i>P. vivax</i>	4	0.2 (0.0-0.4)	60.0
	Mixed	2	0.08 (0.0-.03)	60.0
	Total <i>Plasmodium spp</i> positive n= 2,605	31	1.2 (0.8-1.7)	64.4
nPCR	<i>P. falciparum</i>	80	3.1 (2.5-3.8)	
	<i>P. vivax</i>	10	0.4 (0.2-0.7)	
	Mixed <i>P. falciparum</i> + <i>P. vivax</i>	3	0.1 (0.0-0.4)	
	<i>P. malariae</i>	2	0.07 (0.0-0.3)	
	Total <i>Plasmodium spp</i> positive	87	3.3 (2.7-4.1)	
Seropositivity (n=1,354)	<i>P. falciparum</i> (MSP_1/AMA_1)	559	41.2 (38.7-43.9)	
	<i>P. vivax</i> (MSP_1/AMA_1)	461	34.0 (31.6-36.6)	
	Mixed <i>P. falciparum</i> and <i>P. vivax</i>	181	13.4 (11.7-15.3)	
	Total <i>Plasmodium spp</i> positive(<i>P. falciparum</i> or <i>P. vivax</i>)	665	49.1 (46.5-51.8)	

*Two samples lack RDT result

Table 10 shows the association between DNA screening results and participants sex, age, elevation, bed net use and seropositivity. Seropositive samples had very high probability of being positive for malaria DNA, aOR 10.0 (95% CI: 3.2-29.4), $P < 0.001$ and living in low elevation areas ($< 2000\text{m}$) was a risk factor for malaria infection with marginal significance, aOR 7.3 (95% CI: 0.1-53.5), $P = 0.052$. Weak associations of DNA screening were observed with sex, age group and bed net use in the study samples (Table 10).

Table 10. Association between nested Polymerase Chain Reaction (nPCR) positivity and participant characteristics (sex, age, elevation, bed net use and seropositivity). Data analyzed for three regions (Amhara, Oromia and Tigray), 2015

	PCR Positive			
	Unadjusted OR (95% CI)	P-value	Adjusted OR (95% CI)	P-value
Sex				
Female	Ref		Ref	
Male	1.3 (0.8-2.0)	0.231	0.8 (0.5-1.2)	0.21
Age (Years)				
<5	Ref		Ref	
5-15	1.2 (0.5-2.9)	0.758	1.3 (0.5-3.4)	0.535
15-25	1.0 (0.3-3.1)	0.974	1.3 (0.4-3.6)	0.702
25-50	0.5 (0.2-1.9)	0.343	0.7 (0.2-3.9)	0.528
≥ 50	0.4 (0.1-3.2)	0.407	0.5 (0.1-3.9)	0.512
Elevation (meters)				
>2000	Ref		Ref	
< 2000	7.3 (0.1-53.5)	0.052	5.0 (0.1-53.7)	0.118
Use of bed net N=1,798				
No	Ref		Ref	
Yes	0.7 (0.4-1.3)	0.267	0.8 (0.4-1.3)	0.345
Seropositivity				
No	Ref		Ref	
Yes	5.9 (2.0-17.1)	0.001	10.0 (3.2-29.4)	0.001

Table 11: Malaria prevalence by nested Polymerase Chain Reaction (nPCR) from Rapid Diagnostic Test (RDT) negative samples by region (Benishangul Gumuz, Amhara and Tigray), 2015.

Region	RDT negative samples	Number of PCR positive samples				
		<i>P. falciparum</i>	<i>P. vivax</i>	<i>P. malariae</i>	<i>P. ovale</i>	Total Positive
Beneshangul Gumuz	668	12	2	2*	0	2.1% (14)
Amhara	944	8	2	0	0	1.1% (10)
Tigray	921	5	1	0	0	0.7% (6)
Total	2533	25	5	2	0	1.2% (30)

* 73 samples were RDT positive and 2 samples (Amhara and Tigray each) were not tested for RDT

Table 11 show 30 additional samples were identified as malaria positive among the RDT negative samples, 25 of the cases were *P. falciparum* and five were *P. vivax*. Almost half of the new positives cases 14/30 were identified from samples collected from Benishangul Gumuz Region. Two *P. malariae* cases were identified from the same region mixed with *P. falciparum*.

Table 12: Percentage concordance of Rapid Diagnostic Test (RDT) and slide positive samples compared to nested Polymerase Chain Reaction (nPCR) by region, 2015.

Region	Percentage concordance	
	PCR versus RDT	PCR versus slide positivity
Benishangul Gumuz	90.7% (39/43)	86.7% (13/15)
Amhara	64.3% (9/14)	77.8% (9/7)
Tigray	68.8% (11/16)	71.4% (7/5)
All	80.8% (59/73)	83.9% (26/31)

Table 12 show about 81% (59/73) samples identified as positive by RDT were confirmed by nPCR. And 83.9% of positive slide samples were confirmed by PCR. Higher agreement between RDT and PCR (90.7%) and PCR and slide positivity (86.7%) were observed in Benishangul Gumuz compared to Amhara and Tigray Regions. In Amhara and Tigray regions agreement with PCR

diagnosis were below 80% (Table 12). However, on a species level RDT over diagnosed *P. falciparum* or mixed infection (Table 9).

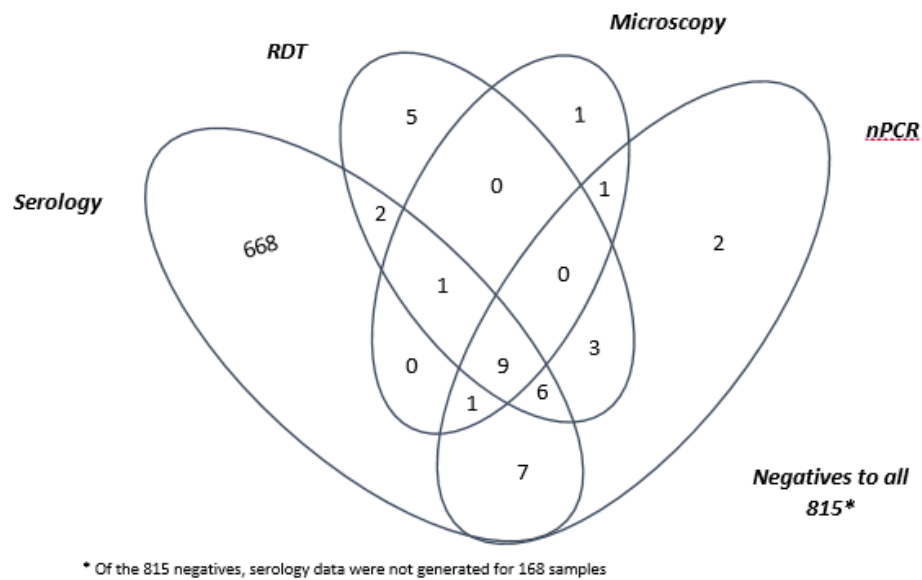


Figure 20: Venn diagram showing the relationships and overlap among serology (IgG MSP-1 or AMA-1), Rapid Diagnostic Tests (RDTs), Microscopy and nested Polymerase Chain Reaction (nPCR) methods (n= 1,522), 2015, samples with complete results collected from Tigray, Amhara and Benishangul-Gumuz Regions of Ethiopia, 2015.

8.3. Determination of G6PD prevalence in Ethiopia

8.3.1. Study population

As depicted in the sample selection flow chart below, of the 11,138 DBS samples 1,998 samples were randomly selected and 1,641 (82.1%) DBS were available for laboratory analysis. DNA were extracted and successfully amplified from 1,548 samples (94.3%). An additional 119 samples were not able to be merged to the original database, resulting in 1,429 merged genotype outcomes and 1,414 with valid genotype results, 15 samples show unsatisfactory gel readings and excluded from the study (Figure 21).

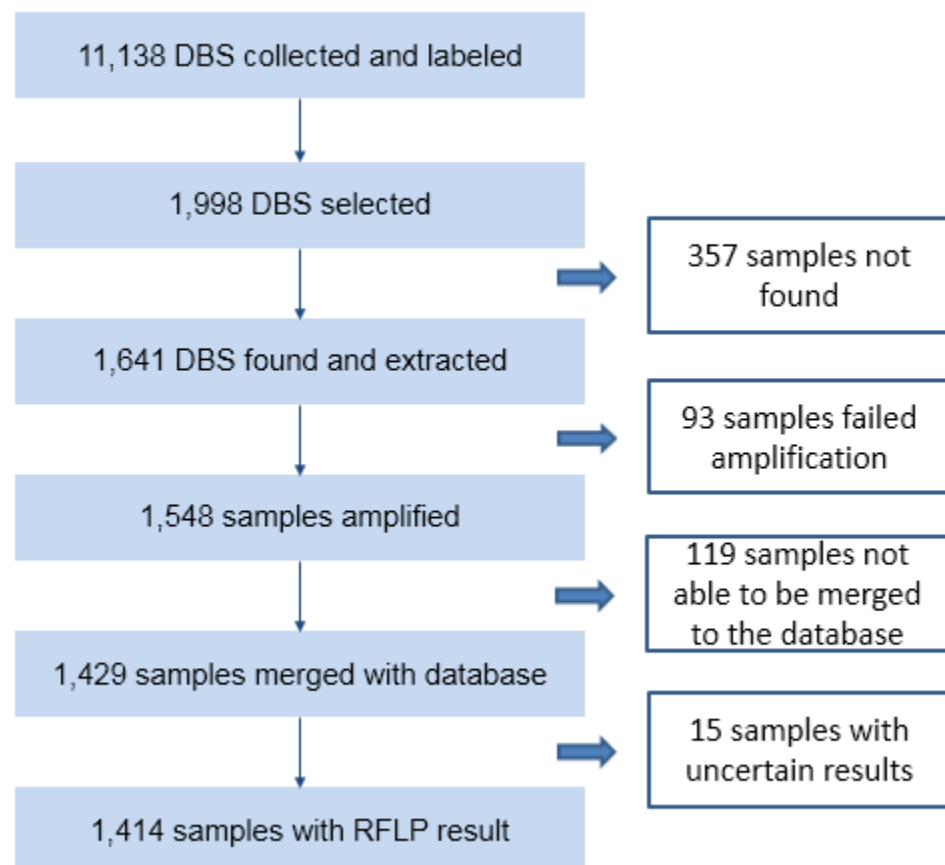


Figure 20. Flow chart for sample selection and processing for G6PD study.

The average age of the study participants was 14.4 (range: 1-89) years. Under five children constitute 51.1% (723/1414) of the participants. More than 60% of the samples were collected from the lower elevation below 2000 masl, of which 53.2% (739/1389) were from an elevation between 1000-2000 masl. Eight percent (111/1389) of the samples were collected from < 1000 masl and the remaining 38.8% (539/1389) were collected from the high land areas >2000 masl. Although samples were randomly selected without considering signs and symptoms of malaria or malaria testing results, 12 samples were positive by malaria RDTs (CareStart™ Malaria HRP-2/pLDH (Pf/PAN) combo), two *P. falciparum*, and five *P. falciparum* or mixed and five were either *P. vivax*, *P. Malariae* or *P. ovale*.

In accordance with the EMIS 2011 planning, representative samples were collected from all the regions of the country. However, for logistic reasons samples collected were lumped for Afar and Somali, Benishangul Gumuz and Gambela and Dire Dawa and Harari. The lumping was made based on geographical and ethnic similarities. Estimates were made therefore based on the above combination.

Table 13 details the number of DBS samples extracted, amplified, and merged for analysis by region. More than 84% of samples were DNA extracted and amplified by region. The success was 100% in Dire Dawa, Harari and Tigray regions, 99.2% and 98.9% in Benishangul-Gumuz and Oromia regions. And 91.3% of the results were merged to the original data base and reported in the current study.

Table 13. Number of DBS samples extracted, amplified, and merged for analysis by region, 2011

Region	No. of samples extracted	No. of samples amplified	No. of samples merged for analysis
Afar & Somali	152	128 (84.2%)	118 (77.6%)
Amhara	302	262 (86.8%)	229 (75.8%)
Benishangul-Gumuz & Gambela	118	117 (99.2%)	109 (92.4%)
Dire Dawa & Harari	11	11 (100%)	10 (90.9%)
Oromia	640	633 (98.9%)	576 (90.0%)
Southern Nations, Nationalities, and Peoples' Region	277	256 (92.4%)	240 (86.6%)
Tigray	141	141 (100%)	132 (93.6%)
Total	1,641	1,548	1,414

Figure 21 show gel electrophoresis photograph with a G6PD mutations, 2011. Arrows in Figure 21 A and B show partial and full digestion for A376G mutations, respectively. Arrows in Figure 21 C show full digestion for G202A (positive control) and Arrow in Figure 21 D show negative control. There was no positive control for C563T (Mediterranean) mutation in the current study.

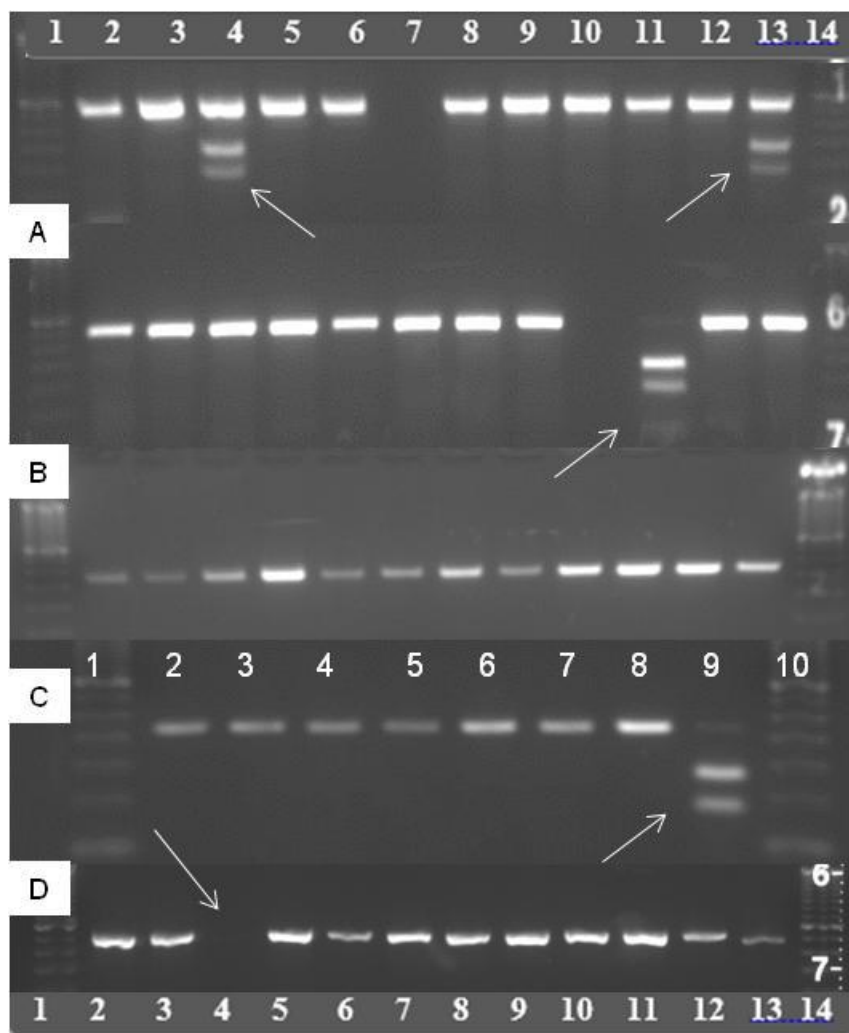


Figure 21. Electrophoresis gel showing a G6PD mutations. First and last lane indicates a molecular ladder of 50 bp. A and B show *A (A376G) mutation. **A.** Lane A4 and A13 show partial digestion, cut size 308 (undigested), 183 and 125. **B.** Lane B11 show full digestion, cut size 183 and 125. **C.** Lane C9 show full digestion for G202A mutation (positive control), cut size 211 (undigested) 81 and 130. **D.** Lane D4 show negative control for C563T mutation, cut size 285 (undigested)

8.3.2. G6PD*A mutations and distribution

Table 14 shows the genotyping results by region. The study showed that the G6PD*A (A376G) mutation was the only mutation found in 8.9% (95% CI: 6.7-11.2) of the analysed samples (Table 13). Of the G6PD*A (A376G) mutations, 57.8% (75/130) was observed in females. The G6PD*A-(G202A) and Mediterranean (C563T) mutations were not detected. No spatial clustering was observed for the G6PD*A (A376G) mutation (Figure 22) which was similar to the spatial distribution of all samples selected (Figure 9 A).

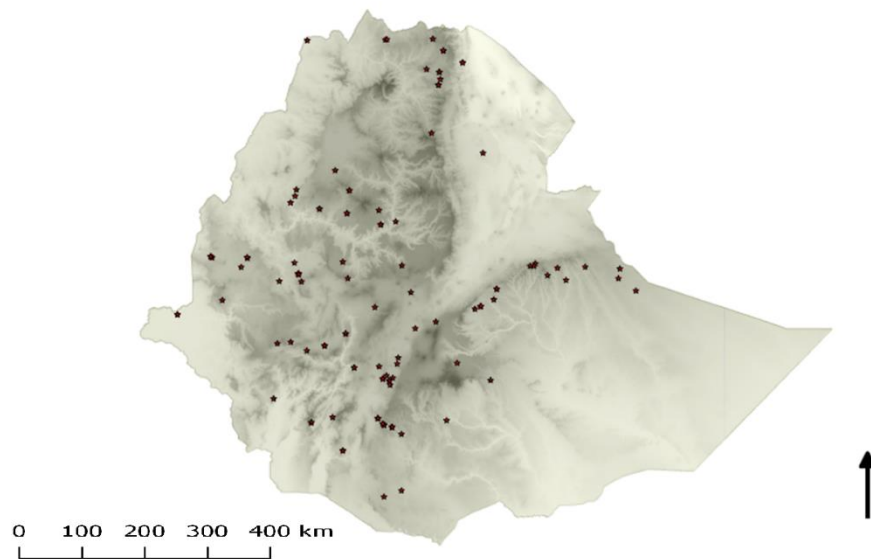


Figure 22. Distribution of households where G6PD*A mutations (n=130) were detected out of the 1998 sampling points across Ethiopia, 2011. (Note: Darker shading represents higher elevation areas, while lighter shading lower elevation areas).

Table 14. Prevalence of three G6PD variants in malaria-endemic areas of Ethiopia, shown by region, 2011.

Region	No. of G6PD*A (A376G)	Weighted Prevalence (%)		
		G6PD*A (A376G) (95% CI)	G6PD*A- (G202A)	Mediterranean (C563T)
Afar & Somali	7	7.7 (0.5-14.9)	0	0
Amhara	15	7.5 (3.0-12.0)	0	0
Benishangul-Gumuz & Gambela	8	5.3 (0.7-10.0)	0	0
Dire Dawa & Harari	0	0	0	0
Oromia	58	8.0 (5.0-10.9)	0	0
Southern Nations, Nationalities, and Peoples'	28	12.2 (5.7-18.7)	0	0
Tigray	14	12.0 (4.4-19.5)	0	0
Total	130	8.9 (6.7-11.2)		

Although not statistically significant, there was regional variation in the prevalence of the G6PD*A (A376G) mutation: the highest prevalence was observed in Southern Nations, Nationalities, and Peoples' and Tigray Regions (12.2%; 95% CI 5.7-18.7 and 12.0%; 95% CI 4.4-19.5, respectively), compared to Dire Dawa and Harari where no G6PD mutations were observed ($P=0.278$). The prevalence of G6PD*A (A376G) was 9.2% (95% CI 6.7-11.8) in areas with altitude less than 2,000 m, and 7.9% (95% CI 2.4-13.4) in areas with altitude between 2,000 and 2,500 m above sea level ($p=0.403$).

9. DISCUSSION

This discussion focusses on major findings of the study and their implications for malaria control and elimination efforts in Ethiopia and beyond. The thesis reports the burden of malaria using advanced serology diagnostic methods (Multiplex serology) in the study settings. Seroprevalence results by age, elevation, region and the spatial distributions are discussed. Modeled seroconversion curves are presented to estimate seroconversion rates over time. In relation to malaria elimination, the identification of subpatent infection; an infection that may not be detected by conventional methods, (microscopy and RDTs) is presented. The role of advanced tools in the detection of clinically less important infections which may play a crucial role in the transmission of malaria is discussed. PCR results are compared with results from conventional diagnostic methods. Finally, PCR-RFLP genotype of G6PD in blood samples collected from national malaria endemic areas of the country are presented. The genotype results show the occurrence and the national and regional distribution of allelic types that indicate G6PD deficiency. The implication of the findings are discussed in relation to the country's efforts towards malaria elimination.

9.1. Malaria sero-epidemiology

Malaria control and elimination efforts require evidence on the magnitude and transmission pattern of the disease (170). Several methods are being employed to explore the disease epidemiology in different settings (37). With declining malaria, in particular, in low transition settings, the effort of determining malaria burden and its transmission pattern is often challenging (80). In Ethiopia, national malaria data are collected by several methods a) the routine Health Management Information System (HMIS), where data are aggregated in woreda/district level and communicated to the Federal Ministry of Health (FMOH) (171) b) the Public Health Emergency Management (PHEM) at the Ethiopian Public Health Institute (EPHI) collect malaria information among the weekly updates on reportable diseases (175). Malaria information at PHEM is further compiled and communicated to the FMOH. Both methods have problems with completeness and in some cases the communicated data can be difficult to trust (53). In addition, the information is gathered from health facilities and may not reflect the disease burden and distribution in communities. c)

National malaria surveys of which the Malaria Indicator Surveys (MISs) are the most notable are methods for measuring malaria burden and distribution. Ethiopia has conducted three consecutive MIS in 2007, 2011 and 2015 (60,61,176). Although, these surveys are the most informative, they have a limitation of being point surveys and may not reflect the true disease burden as discussed in section 11.2

The present study utilized an alternative serology based diagnostic method (Multiplex serology) that may supplement the malaria burden measurements. Malaria seroprevalence stratified by age, elevations and regional categories are reported. Of the 8,749 samples analysed for serology markers, 32.1% had IgG antibodies against combined *P. falciparum* MSP-1 or AMA-1 antigens, and 25.0% for *P. vivax* MSP-1 or AMA-1. The serological results were cumulative antibody responses for the relatively long term markers of transmission, MSP-1 and AMA-1 antigens (80,84,177–179). The relative numbers were lower for children under five years of age at 18.0% for *P. falciparum* and 14.8% for *P. vivax*, suggesting a lower recent transmission in Ethiopia. Seropositivity in children under five years of age may accurately represent recent infection as children have limited exposure time to produce the cumulative antibody response seen in adults (180–182). Over-all, exposure to malaria infection is very high compared to conventional methods and may range up to almost 50% of the adults in the study settings. This contradicts the low prevalence measurement by conventional methods (55,60,62). The RDTs results for the study samples showed 1.8% positivity for *Plasmodium*, of which 0.5% was due to *P. falciparum*.

The study documented significantly higher levels of *P. falciparum* transmission, observed in areas of lower elevation (<2000 m). This is consistent with previous studies (43,46,85), and attributed to the ambient temperature and moisture requirements of the anopheles vector for sporozoite development. *P. falciparum* sporogony requires above 18-20 °C (2,183,184). This reaffirms the national malaria control program's strategy in which high elevation areas (>2000 m) are considered to be areas where malaria transmission is limited and thus not targeted for vector control interventions (53). The evidence of increasing *P. vivax* seropositivity with elevation at least within the range of the study settings, could expand the understanding of malaria transmission in Ethiopia. The relapse of *P. vivax* infections may contribute to the high prevalence of *P. vivax* prevalence observed at higher elevations, besides the occurrence of *P. vivax* sporogony at as low

as 15°C (144,184,185). Seropositivity was high in children under five years of age, particularly in the Gambela and Benishangul Gumuz regions, although it was lower than in adults. A high immune response in children means higher malaria transmission that may need a more intensive and tailored approach of malaria control in the affected regions. A larger proportion of males compared to females were seropositive for both *P. falciparum* and *P. vivax*. Increased malaria risk in men in Ethiopia has been attributed to a greater extent of outdoor, occupational exposure (101,177,180–182).

A total of 8.6% of the individuals were seropositive for *P. malariae* and 3.1% for *P. ovale*. Variable trends of seropositive proportion were observed over the regions for both species. These findings support previous reports of sporadic occurrence of *P. malariae* and *P. ovale* that may be responsible for up to 1% of the malaria incident cases in Ethiopia (77,183,186–188).

Due to the multiplex nature of the serology analysis, antibody cross reaction may be suspected for the reported high prevalence of *P. malariae* and *P. ovale*. However, antibody cross-binding among the different MSP-1 antigens have been documented as non-substantial (158,189). In the present study, to further rule out the effect of cross reactivity or IgG cross-binding, we further analyzed the serology data using an additional conservative approach to define seropositivity (section 5.10.1), thus reporting the most conservative estimates for comparison. The approach, would potentially increase specificity but decrease sensitivity (the two stage conservative approach requires the sample to be considered PmMSP-1 and/or PoMSP-1 positive, individual readings for PmMSP-1 or PoMSP-1 response first had to be above the MFI-bg cutoff and additionally, the PmMSP-1 and/or PoMSP-1 MFI-bg signal for that sample also needed to be above the PfMSP-1 MFI-bg signal for the same sample (ratio to PfMSP-1 greater than 1.0)). By the conservative approach, the overall seropositivity was reduced to 5.1% for *P. malariae* and 1.5% for *P. ovale*, with the trend and pattern of transmission remaining similar. The subpatent study in this thesis discussed below, has analysed selection of study samples from northwest Ethiopia to report positive DNA results for two *P. malariae* out of 2,669 samples tested complementing the serology results. No *P. ovale* cases were detected (section 9.2). However, previous studies have reported sporadic PCR confirmation of *P. malaria* and *P. ovale* transmission in Ethiopia (186,187). Currently, the Ethiopian malaria reporting system doesn't account for *P. malariae* and *P. ovale*.

This may need a revision in the context of malaria elimination and the significant magnitude of transmission reported in the current study. The persistent transmission of *P. malariae* and *P. ovale* has been recently reported in Tanzania (190,191). The malaria elimination effort has little effect in the non-falciparum malaria compared to the significant reduction in *P. falciparum* burden (192)

The use of serological methods in measuring malaria transmission in low transmission settings has been documented in a number of studies and the trends are increasing from time to time (83,84,102,104,164,177,193–199). Earlier studies in Ethiopia (102,177,193) suggested the benefit of serology for large scale national surveys, such as EMIS which documented very low malaria prevalence through RDTs and blood smear (microscopy) methods (60). The earlier estimates from the three EMISs were too low to give detailed information about differences in malaria burden by region or risks of infection due to demographic or behavioral factors.

The current study documented the magnitude of transmission intensity to be highly variable among different regions and elevations in Ethiopia. The results show similar trends as earlier studies: Yalew et al reported a 30.0% and 21.8% seroprevalence for *P. falciparum* and *P. vivax*, respectively, for Amhara region in 2013 (102), the results are in agreement with the current study, we have reported 32.1% for *P. falciparum* and 25.0% for *P. vivax* for the same Region. Several serological studies were carried out in low transmission settings. Our findings are comparable with some of the recent cross sectional serology studies conducted in Somalia (200), Kenya (199), Gambia (198), and Peruvian Amazon (201), where seroprevalences between 19.3% to 36.8% for *P. falciparum* and 17.9% to 33.6% for *P. vivax* were reported. Our findings are lower compared to studies in Uganda (197), Thailand (104), Iran (201), Brazil (195) and Cambodia (196), where above 50% prevalence was reported both for *P. falciparum* and *P. vivax* but higher compared to the low prevalence reported for Myanmar (203); *P. falciparum* at 9.4% and *P. vivax* 3.4%. Some of the variation in seropositivity may be attributed to the different types of laboratory assays used. The challenge of comparing data from ELISA and bead-based multiplex serology assay has been investigated previously (164). Results from multiplex serology are more robust, sensitive and better suited for large-scale surveys compared to ELISA (164). In agreement to our results, heterogeneity of immuno-response for the different antigens were demonstrated in several studies (54,84,200,204). Most studies have used either MSP-1 or AMA-1 antigens. Ashton et al (177)

described MSP-1 antigens to be a better and consistent biomarkers for *P. falciparum*, although Cook et al (84) reported AMA-1 to be better for measuring *P. falciparum* in low-transmission settings. Characterization of antigen markers may be required to further qualify the serology methods (44).

Seroconversion curves can demonstrate the rate and change in malaria transmission over time (84,198,204). We have fitted our data to log likelihood seroconversion models to determine and predict the likelihood of seropositivity over time (age) and predict seroconversion rate (SCR). With some variability among the two antigens used (MSP-1 and AMA-1), heterogeneity was observed on SCR over the different regions, for both species; complementing the heterogeneous transmission and burden reported. Relatively high national SCR per year were predicted for combined MSP-1/AMA-1 antigens 0.084 (95% CI: 0.076-0.091) for *P. falciparum* and 0.049 (95% CI: 0.045-0.055) for *P. vivax*. Although there is limited data to compare national SCR studies, the current finding reported slightly higher SCR compared to a similar study in Amhara region Yalew, et.al, (102). In line with the massive interventions implemented in Ethiopia since 2004/2005 and the declining trend in malaria morbidity and mortality, fitting our data in to a log likelihood model with two forces of infection show more than four and six fold decline in SCR for *P. falciparum* and *P. vivax*, respectively over the past 15 years. However, the changes documented were not statistically significant ($P>0.05$) (Annex. 1). Similar changes in SCR were reported elsewhere and these were evident when there were major changes in transmission due to intervention or other factors (80,84,102).

The heterogeneous fitting of our data to the model across the regions may reflect the geographical variation and programmatic efforts exerted in specific regions. In general, MSP-1 showed higher seroconversion rates compared to AMA-1, both for *P. falciparum* and *P. vivax*. Among the four major regions, Amhara showed highest seroconversion rates; three-fold higher compared to Oromia and two-fold higher compared to Tigray regions, for *P. falciparum* using the MSP-1 antigen. And for *P. vivax* two fold and three fold higher SCR were observed for Amhara compared to Oromia and Tigray regions, respectively. However, the relatively smaller samples analyzed for Amhara Region may require consideration in the comparison of the results. The case build up reported in some places of Ethiopia in 2014 may have impacted on the output of the model and the SCR (API 2014). The variability of the different antigens in fitting the model needs further

investigation. Characterization of specific antigens may be required to monitor change in transmission in a given community.

The seroconversion rates documented nationally, by region and antigens showed the intensity and magnitude of malaria transmission, may be used as a baseline to be compared with future studies that track change in malaria transmission.

Spatial maps developed by Empirical Kriging prediction methods (166) showed areas with high antibody titer to correspond with high malaria burden areas, these were significantly correlated with the API 2014 data, aggregated to woreda/districts. Overall, there was a higher predicted seropositivity in northwest of the country compared to the east for both *P. falciparum* and *P. vivax*.

In the study areas the *P. falciparum* transmission was higher than *P. vivax* transmission. Although data were generated from sampled areas and compared to the API national data, the predicted maps from the serology data showed high correlation with the API maps produced by Empirical Kriging method. The variation in serology data was a challenge in the prediction model. This is documented among the limitations of the spatial analysis.

Similar trends were observed between the serology and the 2014 API data obtained through routine surveillance at FMOH (data not shown). Although routine surveillance can provide crucially important data for decision making for countries with lower transmission, additional biomarkers to track malaria burden may be important as surveillance systems continue to improve and evolve in completeness and quality. Nonetheless, the predicted maps developed are in line with the high malaria burden areas and may be used as an additional input for the malaria control program in the country.

By describing exposure to malaria over a longer period, the serology data better document the magnitude of exposure and the differences in malaria transmission between age groups, regions and elevation. The high seropositivity demonstrated in this study reflects the magnitude of nationwide exposure to malaria over time. This is the first report of its kind in Ethiopia and would serve as credible data that could be used by the National Malaria Control and Elimination Program.

9.2. Subpatent infection

The identification and quantification of subpatent infections is among the important factors in transmission interruption. Recent studies describe symptomatic infections as the tip of the iceberg, showing roughly <5% of the actual on going infection (110,203,205,206). Identifying and treating all forms of infection is a critical requirement on the road towards elimination (173). In this study, microscopy, rapid diagnostic tests (RDTs), serology and nested Polymerase Chain Reaction (PCR) were used to examine the prevalence of infection in low to medium transmission settings in northwest of Ethiopia. Nested PCR identified 30 new positive malaria cases that were missed by RDTs in the community and remain untreated at the time of the survey. The prevalence reported by PCR was 3.3% and was 16.1 %, higher compared to RDT and 64.4% higher compared to microscopy. More than 60% of the PCR positive cases were identified in Benishangul-Gumuz Region, which is one of the high malaria transmission regions in Ethiopia. Benishangul Gumuz region showed a similar high burden of malaria by microscopy, RDTs and serology; confirming the high burden of malaria transmission identified by the NMCP and the need for a tailored approach specific to the area.

Comparison of microscopy and RDT data, with that of nested PCR showed close agreement between the results. Microscopy is considered the gold standard for malaria diagnosis (208), however, it has variable limit of detection (10-100 parasite per microliter of blood) (119). The skill of the technician and the reagents used are determinants of the quality of microscopy, frequently measured as sensitivity and specificity (121). As per the national malaria diagnosis and treatment guideline (171), Ethiopia uses microscopy in health centers and hospitals and RDTs in health posts (primary health care units). In the current study, although the slide preparation and RDT tests were done in the field conditions, where test conditions may not be optimal; the high variability in the detection of infection using the three methods is alarming. On the other hand, the strong association between seropositivity and PCR positivity suggests a role for using seroprevalence results to target and identify areas or hotspots of *Plasmodium* infection.

Recent reports in the detection of HRP2/3 gene deletion may contribute to the lower sensitivity of RDTs (209–211). An ongoing study on the prevalence of HRP2/3 gene deletion in Ethiopia is showing considerable degree of HRP2/3 gene deletion scattered all over the country (Sindew

Mekasha, EPHI, personal communication). As Ethiopia is aiming towards malaria elimination, identification of each and every case will be critical.

Several studies reported subpatent/ sub microscopic or asymptomatic infection in low transmission settings using advanced methods, from different countries (38,110,112,114,116,119,152,203,206,207,212,213). The terms are often used interchangeably to describe a malaria infection that cannot be detected by conventional methods (microscopy and RDTs), but can be detected by more sensitive methods, such as PCR. The synonyms are controversial, some groups prefer to call subpatent infection “chronic malaria infection”, considering their neglected, but important clinical consequences from the patient side (214). The prevalence of subpatent infections in low transmission settings may range from 0.003% to 44%, depending on the sensitivity of tools used and the sample collection area. Studies in Iran (107) and Sri Lanka (111) reported zero prevalence by more sensitive methods, more indicative of no local transmission and evidence of malaria elimination. Sri-Lanka was certified to be malaria-free in 2016 (19). Tadesse et al in 2015 reported a nested PCR prevalence ranging from 1.7% to 5.8% in south west Ethiopia (152). The results are comparable with our findings. In another recent development Girma et al have reported 21.5% prevalence of “asymptomatic” infection in Gambela Region (215). Gambela is the very high malaria burden region neighboring Benishangul Gumuz Region, in southwestern Ethiopia.

Subpatent infection, despite the low parasite density are infectious to mosquitoes (152,216). Studies in Gambia (120), Thailand (119), Peru (108) and Ethiopia (68) showed undetectable asymptomatic malaria infections, to be infectious to mosquitoes which pose a challenge in malaria elimination. A recently published review (217) compared the low density of parasites in low transmission setting to high transmission, and argued that subpatent infections could predict future periods of infection besides their contribution on the infectious reservoir.

Several new tools are being developed with high sensitivity in parasite detection in field settings, some have a sensitivity as low as 22 parasites per mL (Ultra sensitive RDT,uRDT) (40,207). The current study used a sample pooling methodology that decreases cost and is applicable in large sample screening, particularly for low transmission settings (38,218). Most novel methods are yet

to be implemented in field settings. The cost and feasibility of implementing the tests in field settings could limit their wide-scale adoption and uptake in resource-limited countries. Further efforts may be required in domesticating the methods to make them simpler, more cost effective and tailored to local malaria elimination endeavors.

The current study reported a considerable proportion of subpatent infections undetected by the conventional methods. The proportions are higher compared to microscopy and RDTs positivity, but low compared to seroprevalence results. Such subpatent infections could contribute in maintaining transmission in low transmission settings, besides they are causes for chronic anemia, maternal and neonatal mortality among other health problems (214,215). As Ethiopia aims towards malaria elimination, identifying and clearing subpatent infection is critical.

9.3. G6PD prevalence and distribution

According to the WHO malaria elimination perspective, malaria elimination requires the interruption of transmission of all forms and species of malaria parasite from a given geographical area (25). Ethiopia is among the few regions in sub Saharan Africa where *P. falciparum* and *P. vivax* co-exist. *P. vivax* accounts for about 30-40% of incident malaria cases in Ethiopia (12,18,19,77,219,220). A drug capable of interrupting *P. falciparum* transmission and radical cure of *P. vivax*, would greatly accelerate malaria control and elimination efforts. However, only 8-aminoquinolines are the only available drug groups for radical cure (82). The only readily available 8-aminoquinoline drug is Primaquine. Another 8-aminoquinoline drug Tafenoquine, recently completed final clinical trial phases is currently approved by the American Food and Drug Authority to be used in the United States (69). It is yet to be certified by the WHO for global use. Primaquine has been in use for the treatment and radical cure of malaria in Ethiopia. With the global elimination efforts re-initiated, the demand for Primaquine and other 8-aminoquinoline drugs is increasing. However, the widespread use of Primaquine has been limited due to its potential to induce hemolytic anemia in glucose-6-phosphate dehydrogenase (G6PD) deficient individuals (82,139). In Ethiopia, where the national occurrence and distribution of G6PD

deficiency is not clearly documented, determining the G6PD prevalence, distribution, and variants may facilitate the deployment of Primaquine and other 8-aminoquinolines, which will provide the evidence required by the malaria control and elimination program at the Federal Ministry of Health. We have reported the genotypic prevalence of common G6PD deficiency allelic types using samples, representing communities residing in malaria-endemic areas of Ethiopia.

In our study, the more severe variants of G6PD deficiency were not observed. We observed a prevalence of 8.9% of the mild G6PD mutation G6PD*A (A376G); the more severe variants of G6PD*A- (G202A) and Mediterranean (C563T) were absent. Although, the severe type G6PD*A- (G202A) is usually found in a subset of individuals with the G6PD*A (A376G) variant in other studies (140,152,221–223), none of the G6PD*A (A376G) mutant genes in our study carried the G6PD*A- (G202A) mutation. The G6PD*A (A376G) mutation observed in our study is a mild variant, resulting in close to normal (~85%) enzyme activity of a non-deficient person, without significant clinical manifestations of G6PD-related hemolysis (133,161,222,224).

Of the 130 G6PD*A (A376G) samples, most were noted in females, where the expression of the gene is more variable due to lyonization and the overall hemolytic risk was less extensive compared to males. Although not statistically significant, slightly higher G6PD*A (A376G) prevalence was noted in the lower altitude area compared to the higher altitude areas. Less malaria transmission in higher G6PD deficiency prevalence areas in malaria endemic settings, is explained by the selective advantage G6PD deficiency against malaria (221,225).

The Mediterranean (C563T) deficiency were investigated in this study because of the severity of the variant, proximity of Ethiopia to the Mediterranean and Middle East regions, and the ethnic mix observed in country, but this severe variant was not detected in our study. The absence of a positive control for the Mediterranean (C563T) variant was a limitation of our study. However, other recent studies corroborated the absence of the Mediterranean (C563T) variant in Ethiopia (152,223).

Our results are consistent with prior evidence of low G6PD deficiency prevalence in Ethiopia (68,72,117,133,141,153,222,225). For example, the absence of G6PD*A- (G202A) and Mediterranean (C563T) mutations were recently reported from blood samples obtained from malaria patients in southwestern Ethiopia, where 23.3% of the individuals carry the G6PD*A

(A376G) mutation (152,223). However, a study by Carter et al (223) reported several uncommon and new mutations not previously reported in Ethiopia and elsewhere, all with minimal protein effect and no clinical importance. Conversely, a recent study by Shitaye et al (150) has reported 3.5% (7/199) G6PD*A- (G202A) prevalence from a cross sectional school based study in Amhara, Oromia, Gambela and Benishangul Gumuz regions. The study described the mutation from phenotypically normal participants, and may require further investigation.

The current study tested for three of the hundreds of known G6PD variants (224). We cannot definitively conclude that the overall prevalence of clinically relevant G6PD deficient variants is low or excluded the presence of other clinically relevant variants in Ethiopians, especially from regions with very few samples. Our study was not able to conduct phenotypic tests, as we have used stored samples, where enzyme activity is unpredictable. Several sporadic phenotypic studies conducted by different populations failed to report significant presence of G6PD deficiency in Ethiopia. A study by Tsegaye et.al, (227), in Gambela Region (western Ethiopia closer to South Sudan) has reported 0-7.3% phenotypic prevalence of G6PD deficiency using a Carestart G6PD RDT produced by AccessBio. The results of this study was not genotypically assessed. The gold standard in G6PD phenotypic testing is spectrophotometer. The other commonly used testing method is Florescent Spot Testing (FST), which uses visual color change to determine G6PD enzyme activities (as presented in section 4.2 2.5).

Although phenotypic results are not included in the current study, there are some phenotypic G6PD studies that used FST testing method and substantiate our finding: a) A randomized control trial was conducted between 2012 and 2014 in two sites (Bishoftu and Batu) of Oromia region of Ethiopia, to study the efficacy and safety of Arthemeter Lumefantrine and Chloroquine with and without Primaquine in *P. vivax* treatment (228). The study used a FST method for detecting G6PD deficiency to randomize patients in to Primaquine and non-Primaquine arms. Of the 393 screened patients to be enrolled in the study, no G6PD deficient individual was reported. However, intermediate G6PD deficiency was observed in nine (2.3%) patients. The intermediate results were genotypically analyzed for A, A- and Mediterranean mutation and none of them show a positive result for the specified mutations (228). b) A collaborative study between the Ethiopian Public Health Institute, Addis Ababa University and Menzies School of Public Health, Australia recently completed Primaquine dose adjustment study in two sites (Metehara South East and Arbaminche,

South West areas of Ethiopia). The study had a component of evaluating G6PD deficiency using the two common phenotypic tests (FST and spectrophotometer) and genotypic methods (35). Informal communications indicate, of the 580 patients 4 patients showed intermediate G6PD deficiency by FST, the genotype result yet to be reported. c) Another four-arm study that evaluated the therapeutic efficacy of Artemether Lumefantrine and Dihydroartemisinin Piperaquine for *P. falciparum* and Chloroquine and Dihydroartemisinin Piperaquine for *P. vivax* treatment, included radical cure of *P. vivax* with Primaquine at the end of the follow up period (just completed). G6PD was screened for by FST before treatment with Primaquine. One patient was diagnosed G6PD intermediate with FST among 198 screened patients at Arbaminche Health Center in Southern Nations and Nationalities Peoples Region and in Felege Selam Health Center, Metekel zone of in Benishangul Gumuz Region.

The finding of this study was used among the substantial inputs while the MoH recently revise the malaria treatment policy to adopt the addition of single low dose Primaquine for transmission interruption of *P. falciparum* and radical cure of *P. vivax* infections without G6PD testing; but with directly observed therapy and close supervision (229).

Nevertheless, Ethiopia may be a uniquely safe location for the use of Primaquine in the radical cure for *P. vivax* malaria without G6PD testing. The safety of implementing this policy may need to be evaluated through active adverse events and hematological monitoring in the future (75). *P. vivax* case management may evolve with the introduction of short course (7 days) of Primaquine and Tafenoquine (69,76). The development of new point-of-care G6PD deficiency diagnostics and new evidences on the G6PD mutation varieties and distribution may impact current approaches and policies in the future (35,74,228). The current results support the cognizant use of Primaquine for radical cure of *P. vivax* without G6PD testing.

10. VALIDITY AND GENERALIZABILITY

The validity of the study was insured during sample collection, data generation and interpretation. The malaria indicator survey planning followed the Macro MEASURE Demographic and Health Survey Roll Back Malaria (RBM)/Monitoring and Evaluation Reference Group manual. The protocol was adapted to local context and used in the three MISs in Ethiopia. Electronic based data collection was used in order to minimize errors and save time in data transcribing. Questionnaires were sub-headed with local languages. Software that changes dates from local calendar to Gregorian calendar was installed in data collector tools to minimize errors in date related data collection. Local events and major activities, such as holidays were used to minimize recall bias during demographic data collection. Malaria varies with elevation in Ethiopia, areas below 2,000m are generally considered endemic and considered for program implementation. Since, EMIS sampling used enumeration areas (EAs) from the Central Statistics Agency (CSA) and the EA maps did not have elevation data, and thus the preparation of sampling frame based on altitude was a challenge. This challenge was addressed by GIS experts. we have used a 20x20m resolution satellite image data and transposed on CSA digital EA maps for elevation based selection and a mechanism was created for an EA to be considered in the sampling frame i.e. if at least 75 percent of the EA area falls within the required altitude range, the EA will be included in the sampling frame.

Data collectors were intensively trained for two weeks in usage of electronic data collection tools, questionnaires and biological sample collection. A pilot study was conducted to evaluate data collectors understanding. Stringent supervision was organized between the central team and regional offices. Data were actively collected from the sites, where data collectors send the data collected to a server at the EPHI in real time every day. Random data were plotted on map using GPS information and compared with where about of data collectors to rule out fake data collection.

DBS samples were properly dried immediately stored on freezers in nearby health centres before they were transferred to EPHI for deep freezer (-20⁰C) storage until use. Positive and negative controls were used in the assays and inappropriate and unsatisfactory results were repeated. The PI was trained in the use of laboratory methods. Data were generated on standard laboratories and/or compared (EPHI/CDC) with results in certified laboratories (CDC). Similar training was

given for professionals who supported laboratory based data collection. In the serology study, where *P. malariae* and *P. ovale* were observed, a mechanism was developed to minimize the effect of cross-reactivity between species-specific MSP-1 antigens. Additional conservative two-stage approaches that may increase specificity, but decrease sensitivity of the antigen response were used.

Standard statistical methods were used in summarizing, comparing data as well as evaluating associations. Statistical significance above 0.05 and 95% confidence were used where appropriate. Results were discussed and repeated with specific expert groups and interpretations were commented. Up-to-date software and the latest possible shape files were used in data analysis and mapping. Data presented for national and regional levels were weighted with the respective weights developed by the CSA to ensure population representativeness.

The national representativeness of the data ensures the generalizability of the study findings. Representative samples proportional to population size were used and most data were generated at national, regional and on elevation stratifications. Therefore, results can be directly used by the national and regional policy makers without further adjustments. Recommendations were thoroughly discussed with expert groups, presented in local and international conferences and either published or submitted to peer review journals. Besides, our results and recommendations may be generalized and utilized in low to medium malaria transmission settings elsewhere.

11. STRENGTH AND LIMITATION

11.1. Strength

The study had several strengths a) the major strength of the study was its national representativeness. Data were generated based on biological samples collected during a nationwide survey. The study utilized data and sample collected during the Ethiopian Malaria Indicator Surveys (EMISs) conducted in 2011 and 2015 (55,60). The studies covered all malaria endemic areas of the country. Therefore, results and recommendations are practically representative and may be utilized easily by policy makers and other stakeholders b) the hypothesis and rationale of the study were formulated based on current knowledge gap by the national malaria control program in Ethiopia. The student closely works with malaria control and elimination program and the issues covered are among the major challenges of the program that needed to be covered by operational research. In this regard, results from objective three of the study were communicated to the Ministry before the paper was published and this thesis was compiled. As it was critically required for decision making c) the study utilized laboratory based molecular and diagnostics methods to generate data. Molecular epidemiology is a growing field that gives another dimension to the classical epidemiology studies and address some issues that may be overlooked by classical epidemiology. d) New and advanced methods were used to generate data for the current study. Collaborations among Addis Ababa University, EPHI, and Centers for Disease Control (CDC), London School of Hygiene and Tropical Medicine (LSHTM) and Mahidol Oxford Research Unit (MORU) were utilized to access high technology facilities, to secure reagents required, to quality control and compare some the results obtained locally. The collaborations were exploited to solicit expert advice and consultation on the specific topics, results and interpretations, hence, increasing the credibility of the data.

11.2. Limitation

The study limitations are discussed separately by each objective and/or papers:

For objectives one and two, sero-epidemiology study, 35% of DBS samples from the EMIS-2015 survey were not available for serological analysis. Although no systematic bias was introduced,

the representativeness of this sample at the national and regional levels should be interpreted cautiously. There is no well characterized antigen marker that may be utilized in the local context and there were some variations on the immune response for the different antigens used. The multiplex serology method used in this study is a new approach compared to the standard serology method (ELISA), although the method is robust and sensitive it has some limitations in specificity compared to ELISA. Therefore, comparison between Multiplex and ELISA results need to be interpreted carefully. The data used for Bayesian kriging were insufficient to produce refined spatial heat maps and the maps produced show only general trends.

For the third objective: we used samples collected only from three regions owing to limited resources, Tigray, Amhara and Benishangul Gumuz in order to determine subpatent infection. We assumed the regions may represent low to high malaria eco-epidemiology in the country, based on the serology results. However, cautious interpretation must be made in generalizing the results to the national context.

For the fourth objective: the study only tested for three common variants of the hundreds of known G6PD variants and there was no phenotypic data to support the genotypic results. Therefore, our results cannot definitively conclude to report the absence of all clinically relevant variants from Ethiopian, especially from regions with very few samples. There was no positive control for Mediterranean type of mutation for quality control. The current study had a plan to look for possible spatial correlation between G6PD deficiency and seroprevalence, however, the absence of the major deficient mutations and the random distribution of the clinically insignificant G6PD *A type mutation forced us to drop this assessment.

12. CONCLUSION

Following the profound decline of malaria morbidity and mortality in the past two decades, the National Malaria Control and Elimination Program in Ethiopia has set a vision of eliminating malaria step by step from low to high transmission areas. The plan is included on the current Strategic Plan (2015-2020) and a road map document that outlines the detailed approach of elimination. Malaria elimination is not a new endeavor for Ethiopia. Ethiopia was part of the global malaria eradication program from 1950s to 1970s. Ethiopia was not successful in eliminating malaria at that period and went back to malaria control on early 1970s. Now, following the malaria control gains, eliminating malaria from national borders with eventual global objectives of complete eradication is back on the table. Ethiopia is in now on a cross road similar to the 1960's. In order to complement and produce evidence that may be used as an input towards the national malaria elimination effort, this PhD thesis work used advanced diagnostic and epidemiological methods to explore the study questions.

The study used multiplexing methods and serological markers to measure malaria exposure burden and transmission intensity of the four human malaria species. The current study documented heterogeneity in malaria burden and transmission over differing elevations stratifications, age groups and administrative regions. Malaria exposure was by far higher compared to the active infection reported by microscopy and RDTs. Lower elevations have high malaria burden compared to the highlands with notable difference within the major two species, *P. falciparum* and *P. vivax*. *P. falciparum* prevalence increases with decreasing elevation, whereas *P. vivax* increase with elevation in the study settings up to 2,500 m; showing *P. vivax* is more of a “highland malaria” compared to *P. falciparum*. Major variability was observed in the spatial distribution and dynamics of transmission over the regions. The northwestern part of the country, carrying the largest burden compared to the East. Among the regions, Gambela and Benishangul-Gumuz regions documented the highest burden in malaria transmission. Generally, people in older age groups show high exposure to malaria, but importantly children under five years of age depict low level of malaria exposure in agreement with the current reports of declining malaria morbidity and mortality. The current study has also documented a previously unrecognized relatively high transmission of *P.*

malariae and *P. ovale*. Overall, from the sero-epidemiology study, the relative high transmission in the Gambela and Benishangul-Gumuz regions, the increase of *P. vivax* seropositivity with elevation, and the notable presence of *P. malariae* and *P. ovale* in all administrative regions (given *P. ovale* possessing hypnozoite stage) may require programmatic attention in malaria control and elimination endeavors. The seroprevalence results may be used as baseline data for the recent malaria elimination efforts and may further help the NMCP in tailoring intervention approaches.

Given the very high variability, malaria epidemiology in Ethiopia has been recognized as a low transmission setting with periodic case-build up and epidemics. Among the major factors of the transmission dynamics, the subpatent infection circulating in the population is substantial. The current study reported a considerable burden of subpatent infections in the study area. Again, with regional variation, our study has documented almost a threefold subpatent infection undetected by microscopy which remains untreated circulating in the community. Subpatent infections are infectious to mosquitoes, contributing for malaria transmission in addition to their debilitating chronic effect on the affected individuals. Countries setting up for malaria elimination in low transmission settings need to consider low density or subpatent infection management as this may derail the effort towards malaria elimination. However, the magnitude of subpatent infection we report here contradicts the iceberg assumption of subpatent infection in low transmission settings.

In our study, the more severe variants G6PD *A- (G202A) and Mediterranean (C563T) mutations were not observed. The G6PD *A (A376G) mutation observed is a mild variant, resulting in close to normal (85%) enzyme activity of a non-deficient person, without significant clinical manifestations of G6PD-related hemolysis. Our results are consistent with the limited historical evidence of low G6PD deficiency prevalence in Ethiopia. As this study, only tested for three of the hundreds of known G6PD variants, we cannot definitively conclude that the overall prevalence of clinically relevant G6PD deficient variants is zero and to definitively support the use of Primaquine for radical cure of *P. vivax*, without G6PD deficiency prior testing. Nonetheless, the likely very low risk of hemolysis, especially in the major ethnic groups of Ethiopia, suggests the inclusion of Primaquine use in the national treatment guidelines. The risks and benefits of Primaquine radical cure without G6PD testing will need to be further assessed in Ethiopia as the

P. vivax case management landscape expected to evolve and may incorporate higher dose and shorter course of Primaquine and Tafenoquine in the short run.

The national malaria control and elimination program in Ethiopia recently revised its malaria treatment guideline by re-introducing the use of Primaquine for transmission interruption of *P. falciparum* and radical cure of *P. vivax* as directly observed therapy with close follow up. The results of the current study were communicated to the MoH and substantially contributed during the informed decision making process, i.e. revising the national malaria treatment guideline to re-introduced Primaquine radical treatment with out prior G6PD testing.

In summary, the current thesis work used advanced diagnostic and molecular tools to produce credible evidences required on the epidemiology and risk factors of malaria relevant for malaria elimination. The information generated may help the national effort towards eliminating malaria. The study also highlited the need for assessing and introducing advanced tools, such as multiplex serology and to accelerate the effort towards malaria elimination.

13. RECOMMENDATIONS

13.1. Program/policy recommendation

The thesis work was planned and organized to produce evidence that may help the National Malaria Control Program to further consolidate its effort on malaria elimination.

13.1.1. Malaria sero-epidemiology study

- Malaria transmission and the burden of the disease is much higher in Ethiopia compared to the report by conventional methods (Microscopy and RDTs), this suggests the need for the use of advanced methods in tracking changes in transmission.
- Malaria transmission is heterogeneous, and high transmission is observed in northwest of the country. Particular programmatic interventions are required in Gambela and Benishangul Gumuz regions.
- Generally, low seroprevalence was observed in children under five years of age, suggesting a decline in malaria transmission. As under five children have less exposure time compared to adults, prevalence in children shows the recent burden in malaria transmission, thus may be used as an indicator for the declining trend of malaria transmission in the country.
- Malaria transmission is dictated by elevation but significant variation was observed between *P. falciparum* and *P. vivax* dynamics; there was high transmission of *P. vivax* on the high lands compared to the high transmission of *P. falciparum* in the low lands within the study elevation range up to 2,500m. This may suggest a tailored approach in control activities and resource allocation
- Significant transmission of *P. ovale* and *P. malariae* was observed, given the relapse and hypnozoite stage of *P. ovale*, programmatic consideration is recommended in diagnosis and treatment of *P. ovale* and *P. malariae* on the road towards malaria elimination.
- The demonstrated seroprevalence may be used as baseline in tracking transmission dynamics in the malaria elimination efforts.

- Multiplex serology has been proved to be a useful tool in tracking changes in malaria transmission pattern. Its robust nature, convenience in screening several antigens at the same time and relative low cost may guarantee consideration in future use in relation to malaria elimination.

13.1.2. Subpatent infection

- The significant burden of subpatent infections found circulating undetected in the study area may be generalized to all malaria endemic areas. Subpatent infections have debilitating chronic effect on patients besides they are infectious to mosquitoes and contribute to the transmission of malaria. Controlling subpatent infection may require more emphasis during malaria control and elimination efforts.
- Advanced diagnostic tools, such as PCR that confirmed the occurrence of subpatent infections; treatment strategies, such as single dose Primaquine, radical cure and/or mass treatment may need to be emphasized.
- Although we have reported the presence of subpatent infections, the proportion attributable to subpatent infection was less than the proportion reported from other low transmission settings. Intervention modalities may need to be based on the actual prevalence of subpatent infection for better use of limited resources.

13.1.3. G6PD deficiency and mutations

- The more severe variants of G6PD mutations were not observed, our finding complements the re-institution of radical cure of relapsing malaria without G6PD testing, but with close follow up adopted by the National Malaria Control program in Ethiopia.

13.1.4. Recommendations for further research

- Variability was observed on the immune responses of the multiple antigens used in the current study. Given the potential of multiplex serology on the screening of several antigens in a given run, characterization of antigens that fits local context may be required.
- Dichotomizing serology results is required to harmonize their use with data generated from conventional methods. However, continuous data analysis may be recommended to diversify the scope and depth of the serological data generated.
- Further work is required to customize or produce new models that utilizes the multiplex serology data and predict transmission patterns and change in transmission.
- Further work is required on the prediction and development of heat maps that show the spatial distribution of the disease together with environmental, social and climatic factors.
- Studies are required on the approaches of managing subpatent infection in the context of malaria elimination.
- The sensitivity of uncommon species (*P. malariae* and *P. ovale*) to antimalarial drugs need to be studied.
- The current study, may not conclusively exclude the risk of hemolysis after 8-aminoquinoline treatment. Genotypic studies guided by phenotypic approach, inclusive of all ethnic groups is recommended.
- The advent of Tafenoquine (single dose) and short course Primaquine (7 days, 30mg/kg) for radical cure of recurrent malaria are extra tools towards malaria elimination, but the risk of hemolysis and the related high cost for malaria control program, if prior testing is required, there need to be carefully studied particularly in low resource setting countries.
- The discordance observed among Microscopy, RDTs and PCR diagnostic methods is alarming and need further investigation. This will be more imperative in light of HRP2 deletions observed in Ethiopia and neighboring countries.

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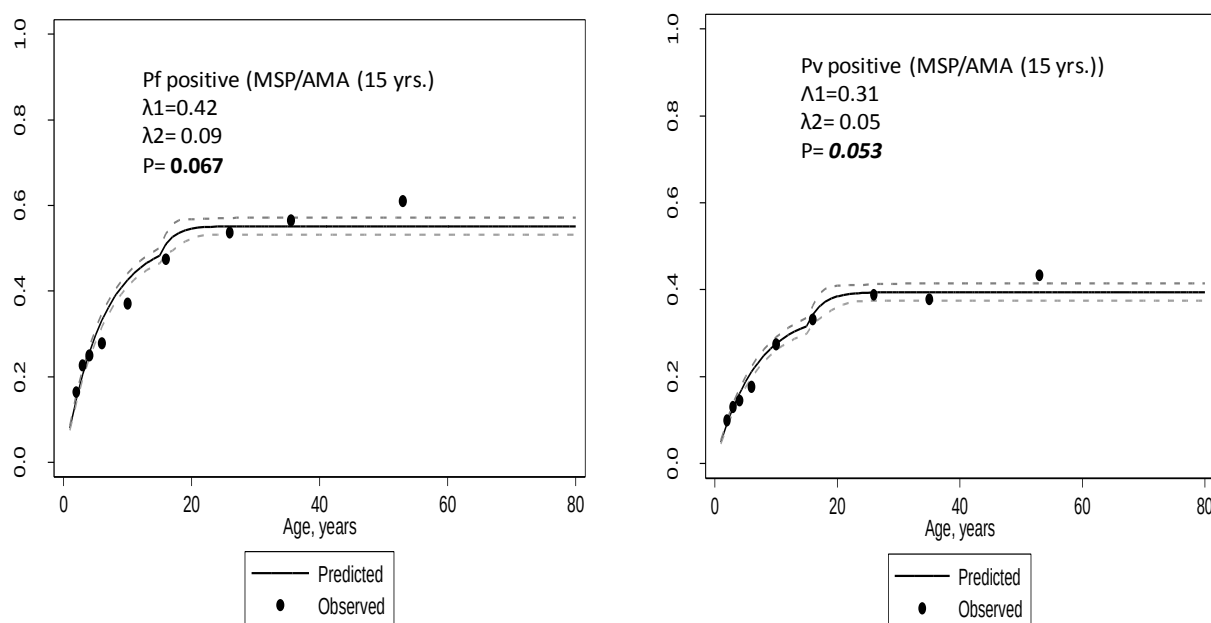
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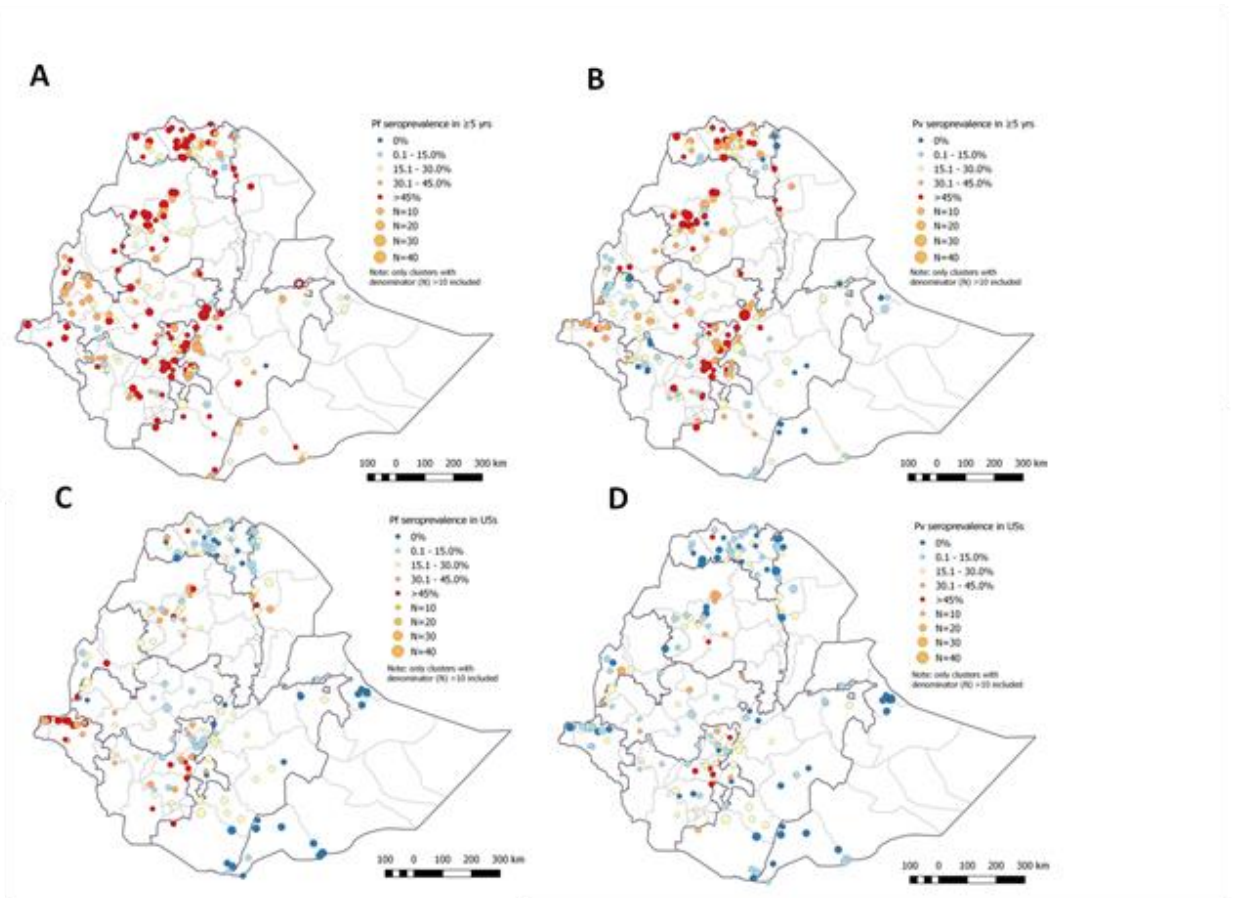
16. APPENDICES

Annex 1. National seroconversion curves



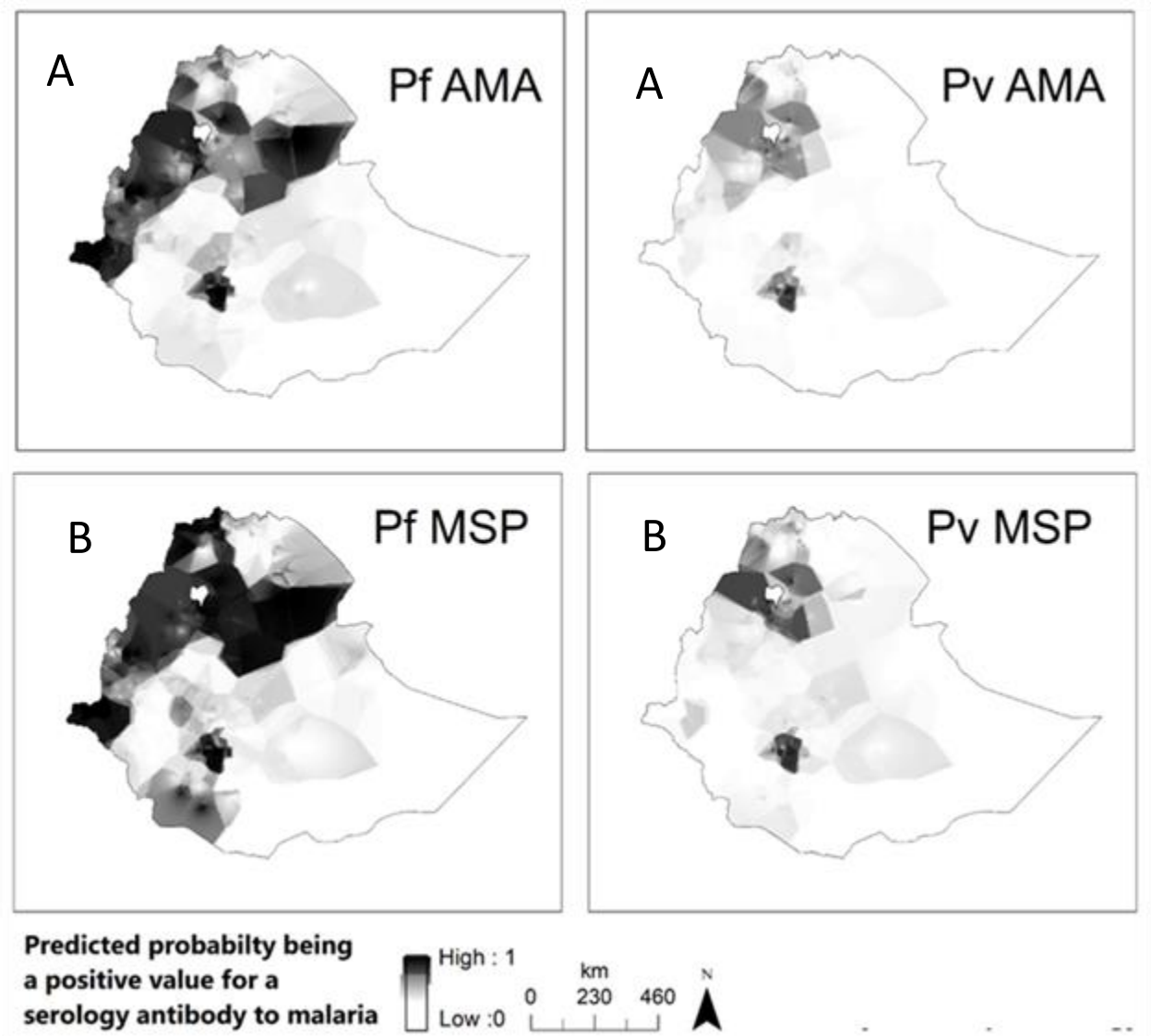
Annex 1: National age-seroconversion plots for antibody responses for *P. falciparum* (MSP-1). Y axis represent probability seropositive over age. Seroconversion curves represent the rate at which a population becomes seropositive to specific antigens resulting in seroconversion rates (SCR) or lambda (λ). In each graph, points represent age seroprevalence (by deciles), unbroken line represents maximum likelihood curves and broken lines represent the 95% confidence interval. The plot represent a more than four and six fold in seroconversion rates for *P. falciparum* and *P. vivax*, respectively over the past 15 years. However, the changes noted were statistically non-significant ($p>0.05$).

Annex 2. Spatial distribution of seroprevalence by enumeration area EA



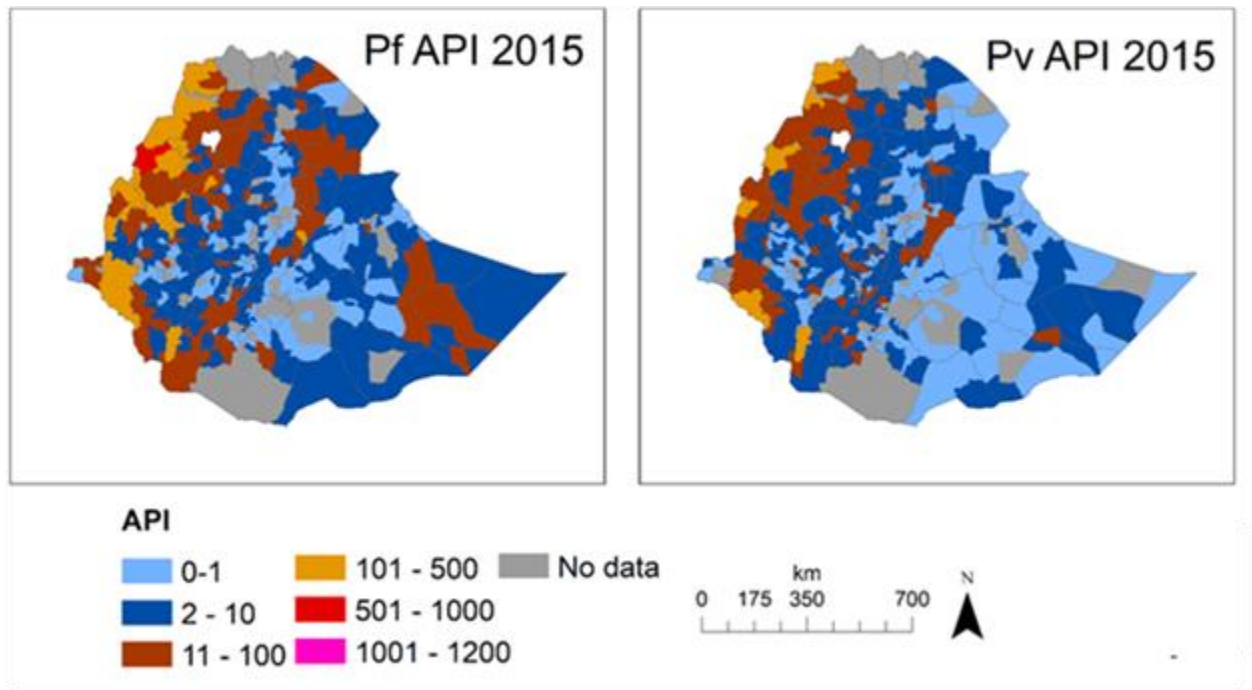
Annex 2. Spatial distribution of seroprevalence for *P. falciparum* and *P. vivax* in malaria endemic areas of Ethiopia (EMIS-2015). Seropositives for either MSP-1 or AMA-1 antigens for *P. falciparum* and *P. vivax* were plotted separately for all ages (A. *P. falciparum* and B. *P. vivax*) and for children under five years of age (C. *P. falciparum* and D. *P. vivax*). Circular size represents number of samples tested per enumeration area (EA), and clusters with less than 10 samples per cluster were excluded.

Annex 3. An Interpolated surface map for *P. falciparum* and *P. vivax* antigen responses



Annex 3. An interpolated surface map for *P. falciparum* antigen responses (A. MSP-1 and B. AMA-1) and *P. vivax* antigen responses (A. MSP-1 and B. AMA-1). Darker color shows high probability of being exposed to malaria infection (above the cut-off for seropositivity) and lighter color lower, 2015.(Maps were created by MORU Epidemiology unit, Empirical Bayesian kriging(169))

Annex 4. An interpolated surface map for *P. falciparum* and *P. vivax* API, 2014.



Annex 4. An Interpolated surface map for *P. falciparum* (left) and *P. vivax* (right). Empirical Bayesian kriging was used to produce the maps from the Annual Parasite Incidence data in Ethiopia (API 2014). Maps created by MORU Epidemiology unit.

Annex 5. Consent & Assent forms

A. Malaria Indicator Survey Participant Consent form: for household interviews and women's questionnaire

- a. Age 19 years or older; and parent/legal guardian of women age 12-18 years eligible for women's questionnaire

Introduction

Greetings, my name is _____ and I work with the Ministry of Health.

If you are the legal guardian of a child who is being asked to participate, the term “you” used in this consent refers to your child.

You are being asked to be a part of this survey. This form is designed to tell you everything you need to think about before you decide to consent (agree) to be in the survey or not to be in the survey. It is entirely your choice. If you decide to take part, you can change your mind later on and withdraw from the survey. You can skip any questions that you do not wish to answer.

Before making your decision:

- Please carefully listen as this form is read to you
- Please ask questions about anything that is not clear

You can take a copy of this consent form, to keep. Feel free to take your time thinking about whether you would like to participate. By agreeing to participate you will not give up any legal rights.

Survey Overview

We are visiting with household in your community and other communities in Ethiopia to find out about malaria. There will be a total of 13,880 households in this survey. The purpose of the survey is to assess use of mosquito nets and household spraying against mosquitoes. We will also ask questions about your family, including illnesses and deaths, and your use of health services. Your responses will allow us to further understand how big a problem malaria is in your area, and how well malaria control is working.

If your household agrees to participate, we may separately ask the family members individually if they will allow us to collect some of their blood to test for malaria.

Procedures

I would like to talk with you for about 30 minutes and ask a few standard questions. If you have mosquito nets we will ask to look at those nets. We will ask about births, deaths and treatment of recent illnesses in children.

Risks

There are no known risks from participating in the household interview except for possible emotional upset when talking about illness and death of family members.

Benefits

There are no direct benefits to you or your household for participating in this survey. This survey is designed to learn more about malaria. The survey results may be used to help others, including you and your household, in the future. You will be able to receive all benefits or assistance from any future program even if you refuse to participate in this interview.

Compensation

You will not be offered payment or compensation for participating in this survey.

Other options outside this Survey

If you decide not to enter this survey, there is care available to you through your local health facility (health post, health clinic, or hospital).

Confidentiality

A survey number rather than your name will be used on survey records wherever possible. Your name and other facts that might point to you will not appear when we present this evaluation or publish its results.

Certain offices and people other than the survey team may look at survey records. Government agencies and participating Institutional Review Board employees overseeing proper survey conduct may look at your survey records. These offices include the Offices for Human Research Protections, the Institutional Review Boards, the Offices of Research Compliance and the Offices for Clinical Research. Study funders may also look at your survey records. The study team will keep any records we create private to the extent we are required to do so by law.

Survey records can be opened by court order. They may also be produced in response to a subpoena or a request for production of documents.

Voluntary Participation and Withdrawal from the Survey

Your participation is entirely voluntary. You may refuse to do any procedures you do not feel comfortable with, or answer any questions that you do not wish to answer.

Contact Information

Contact the Ethiopian Public Health Institute (EPHI), Dr Amha Kebede or Mr. Ashenafi Assefa at + 251 112 751555.

- if you have any questions about this survey or your part in it,

- if you have questions, concerns or complaints about the survey

Contact Ethiopian Public Health Institute, Mr. Ashenafi Assefa at+ 251 112 751555:

- If you have questions about your rights as a survey participant.
- If you have questions, concerns or complaints about the survey.
- You may also let the IRB know about your experience as a survey participant through our Survey Participant Survey at <http://www.surveymonkey.com/s/6ZDMW75>.

Consent

Do you agree to participate?

Thank you for your time.

b. Verbal informed assent for women's questionnaire (age 12-18 years)

Greetings, my name is _____ and I work with the Ministry of Health.

We are asking you to volunteer to be in an evaluation survey. You or someone in your family may be sick with malaria. The purpose of the survey is to learn about mosquito net use and household spraying against mosquitoes. We will also ask questions about your family, including sicknesses and deaths, and your use of health services.

There are no known risks from participating in the interview except for possible emotional upset when talking about illness and death of family members. There are no direct benefits to you or your household for participating in this survey. The survey results may be used to help others, including you and your household, in the future.

You can refuse (say no) to be in this survey. Your doctors or your parents cannot make you be in the survey if you don't want to be in it. If you refuse, there is care available to you through your local health facility (health post, health clinic, or hospital). You may refuse to do any procedures you do not feel comfortable with, or answer any questions that you do not wish to answer.

The survey team will talk to you about what it means to be in a survey. He or she will also talk to you about what it means to have malaria. You should ask the interviewer all of the questions you have. You should also talk to your parents about the survey and about malaria.

Do you agree to participate? *(If the child aged 12-18 years declines assent, the parent/legal guardian cannot force them to participate.)*

Thank you for your time.

- c. Verbal informed consent for malaria and anemia test: Age 19 years or older; and parent/legal guardian of children less than 18 years

Introduction

Greetings, my name is _____ and I work with the Ministry of Health.

If you are the legal guardian of a child who is being asked to participate, the term “you” used in this consent refers to your child.

You are being asked to be in a survey. This form is designed to tell you everything you need to think about before you decide to consent (agree) to be in the survey or not to be in the survey. **It is entirely your choice. If you decide to take part, you can change your mind later on and withdraw from the survey. You can skip any questions that you do not wish to answer.**

Before making your decision:

- Please carefully listen as this form is read to you
- Please ask questions about anything that is not clear

You can take a copy of this consent form, to keep. Feel free to take your time thinking about whether you would like to participate. Do not agree to participate unless you have had a chance to ask questions and get answers that make sense to you. By agreeing to participate you will not give up any legal rights.

Survey Overview

We are visiting with heads of household in your community and other communities in Ethiopia to find out how much malaria there is in your area. We want to evaluate the impact of the national malaria control program once it has been implemented in your area. There will be approximately 23,443 participants in this survey tested for malaria, including 9,058 children under-five years of age also tested for anemia.

Procedures

You (or your child) live in a household that has been selected at random for a survey and for a blood test of some or all household members. A finger-prick to draw seven drops of blood will be done by a nurse with a sterile lancet in the same way that they do it at the clinic. Four drops of blood will be put onto a microscope slide for analysis in the laboratory to determine if you have malaria; one drop of blood will be put onto filter paper for possible analysis to determine if you currently have malaria (by polymerase chain reaction [PCR] detection), or have been exposed to malaria in the past (by serology), and one drop of blood will be used for a rapid diagnostic test for malaria, which will be conducted in your presence. If your child is ≤ 5 years of age, a test for anemia will also be done on another drop of blood. The results of the rapid diagnostic test and the anemia tests will be given to you immediately, but you will not receive results from the microscope slide analysis or other laboratory tests, since these results will not be available in a timely manner.

Risks

The finger-prick will be momentarily uncomfortable. The drugs used to treat malaria and anemia are safe and effective, but any drugs can cause side effects in a small number of people.

Benefits

If you or your child is diagnosed with malaria or anemia, you will be offered treatment according to Ministry of Health guidelines. If you are pregnant, you will be referred for malaria treatment that will not harm the baby. If you or your child is very sick, or your child is very short of blood (severely anemic), you will be referred to the nearest health facility for urgent treatment.

This survey is designed to learn more about malaria. The survey results may be used to help others, including you and your household, in the future. You will be able to receive all benefits or assistance from any future program even if you refuse to participate in this interview.

Compensation

You will not be offered payment or compensation for participating in this survey.

Other options outside this Survey

If you decide not to enter this survey, there is care available to you through your local health facility (health post, health clinic, or hospital).

Confidentiality

A survey number rather than your name will be used on survey records wherever possible. Your name and other facts that might point to you will not appear when we present this survey or publish its results.

Certain offices and people other than the survey team may look at survey records. Government agencies and participating Institutional Review Board employees overseeing proper survey conduct may look at your survey records. These offices may include the Office for Human Research Protections, the Institutional Review Boards, the Offices of Research Compliance and the Offices for Clinical Research. Survey funders may also look at your survey records. The survey team will keep any survey records we create private to the extent we are required to do so by law.

Survey records can be opened by court order. They may also be produced in response to a subpoena or a request for production of documents.

In Case of Injury

If you get ill or injured from being in the survey, the survey organizers would help you to get medical treatment. Partner institutions and Ethiopian Public Health Institute (EPHI) have not, however, set aside any money to pay you or to pay for this medical treatment. The only exception is if it is proved that your injury or illness is directly caused by the negligence of a survey or sponsor employee. “Negligence” is the failure to follow a standard duty of care.

If you believe you have become ill or injured from this survey, you should contact the Ethiopian Public Health Institute (EPHI), Mr Ashenafi Assefa at + 251 112 751555. You should also let any health care provider who treats you know that you were part of an evaluation survey.

Costs

There will be no costs to you for participating in this survey. You will not be charged for any of the survey activities.

Voluntary Participation and Withdrawal from the Survey

Your participation is entirely voluntary. You may refuse to do any procedures you do not feel comfortable with, or answer any questions that you do not wish to answer.

Contact Information

Contact the Ethiopian Public Health Institute (EPHI), Dr. Amha Kebede or Mr. Ashenafi Assefa at + 251 112 751555:

- if you have any questions about this survey or your part in it,
- if you feel you have had a survey-related injury or a bad reaction to drug treatment
- if you have questions, concerns or complaints about the survey

Contact Ethiopian Public Health Institute, Mr. Ashenafi Assefa at 251 112 751555:

- If you have questions about your rights as a survey participant.
- If you have questions, concerns or complaints about the survey.
- You may also let the IRB know about your experience as a survey participant through our Survey Participant Survey at <http://www.surveymonkey.com/s/6ZDMW75>.

Consent

Do you agree to participate?

Thank you for your time

d. Verbal informed assent for malaria test (for children 6 - 18 years)

Greetings, my name is _____ and I work with the Ministry of Health.

We are asking you to volunteer to be in a program evaluation survey. You may be sick with malaria. The purpose of this evaluation is to determine if you have malaria.

We will prick your finger one time to collect a several drops of blood the same way that they do it at the clinic. We will put the blood on a glass slide, on a piece of paper, and on a malaria rapid diagnostic test. These tests will tell us if you currently have malaria, or have had it in the past. Your finger may hurt for a few minutes after the finger prick. If you have malaria we will give you medicine to treat you.

You can refuse (say no) to be in this survey. Your doctors or your parents cannot make you be in the survey if you don't want to be in it. If you refuse, there is care available to you through your local health facility (health post, health clinic, or hospital). You may refuse to do any procedures you do not feel comfortable with, or answer any questions that you do not wish to answer.

The survey team member will talk to you about what it means to be in a survey. He or she will also talk to you about what it means to have malaria. You should ask the interviewer all of the questions you have. You should also talk to your parents about the survey and about malaria.

Do you agree to participate? ***(If the child aged declines assent, the parent/legal guardian cannot force them to participate.)***

Thank you for your time.

Annex 6. Laboratory procedures

Serology study

Luminex Serology: Antibody Coupling Kit (xMAP®)

<https://www.luminexcorp.com/antibody-coupling-kit/>

The xMAP® Antibody Coupling Kit, or “AbC” kit, is a reagent kit that contains everything a researcher needs to couple an antibody to MagPlex® Microspheres. Similar to an ELISA plate, MagPlex Microspheres can be used as the substrate on which immunoassays can be performed. Unlike a traditional ELISA, however, this assay method requires much less antibody, sample, and time; with equal or better sensitivity and dynamic range.¹

Since each microsphere is uniquely addressable, multiple immunoassays can be performed simultaneously with the same sample, in the same well. Once your desired antibody is coupled to the surface of the bead you can begin optimizing monoplex or multiplex immunoassays to suit your needs.

Experience the benefits of immunoassays on xMAP® Technology, including:

- Less sample
- Less antibody (up to 90% less antibody in some cases²)
- Less labor
- Greater reproducibility
- Equal or better sensitivity and dynamic range

Benefits

- Conveniently includes all reagents and consumables necessary to couple antibodies to MagPlex microspheres
- Easy to use protocol

Recommended Complementary Products

- MagPlex Low Concentration Microspheres (MC100XX-ID) are the perfect amount of beads for assay development
- The Luminex Magnetic Tube Separators (CN-0288-01) are ideal for executing the wash steps required in bead coupling

Specifications

Kit Contents

- Easy to follow coupling protocol, including guidance on how to assess coupling efficiency
- Pre-optimized reagents and buffers, including EDC³
- All required consumables (i.e. reaction tubes and transfer pipettes)

Run Times

- Hands-on time = ~40mins

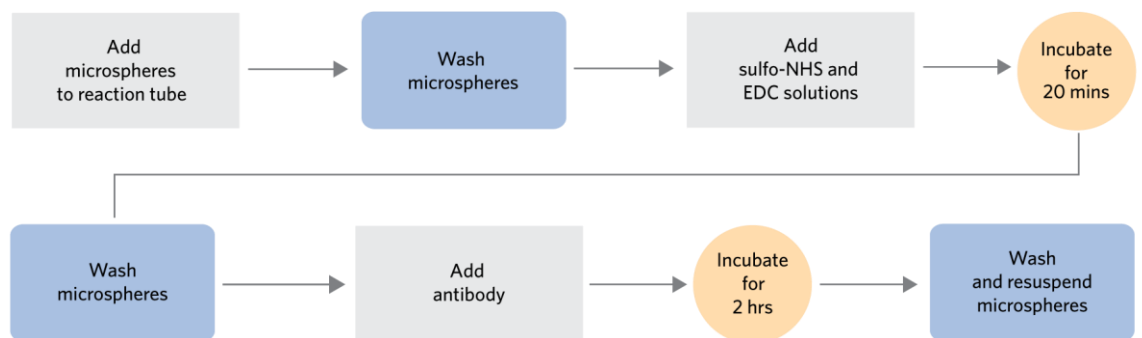
- Total turn-around time = ~3hrs

Kit Capacity

- Small scale: up to 10 reactions of 5×10^6 beads/reaction or less
- Large scale: up to 4 reactions of greater than 5×10^6 beads/reaction
- Maximum scale is 12.5×10^6 beads/reaction

Number of Beads that can be Coupled per Kit

- Maximum: 50×10^6 beads (4 reactions @ 12.5×10^6 beads/reaction)
- Minimum: 2.5×10^6 beads (1 reaction @ 2.5×10^6 beads/reaction)



Workflow: xMAP Antibody Coupling Kit Workflow Overview for Use with MagPlex Microspheres:

Manual MagPlex® Bead Washing

Not using the most efficient way to wash your beads can often lead to lost sample and beads—something we try to prevent! Check out the easy steps below for ways to help your manual MagPlex® bead washing process, and help keep your beads in your plate!

Method

The assay plate is placed on the Luminex Magnetic Plate Separator at the end of an assay incubation step and sits on the separator for one minute to bring all MagPlex Microspheres to the bottom of each well (Images 1 & 2).

Images 1 & 2. Place the plate on the separator for 1 minute.

Clip the 96-well plate on the Luminex Magnetic Plate Separator, both the plate and the magnetic plate separator are rapidly and forcefully inverted over a sink or biohazard receptacle to evacuate the liquid reagent from the wells (Images 3 & 4).

Images 3 & 4. Rapidly and forcefully invert the plate and separator.

The rapid, forceful inversion motion over the sink or biohazard receptacle must be repeated two to three times in quick succession to effectively and sufficiently remove the liquid reagent from the microspheres in each well. You may see the MagPlex Microspheres at the bottom of the well depending upon the plex and concentration in the assay (Image 5).

Without turning the plate over to ‘peek’ at the wells **BLOT** on a paper towel before turning the plate over (removes additional liquid reagent). Work in a timely fashion to not let the beads dry out.

Image 5. MagPlex Microspheres may be seen at the bottom of wells following liquid evacuation.

Once the assay plate is removed from the magnetic separator, liquid reagent can be added back to each well as a part of a wash step or to proceed with the next reagent addition step in the assay protocol.

This step is **NOT** performed on the magnetic plate. If you have any question, please contact your Field Applications Specialist or Luminex's Technical Support Team.

PCR based RFLP

Protocol for DNA Purification from DBS (QIAamp DNA Mini Kit) (Ref: Handbook for Qiagen QIAamp®96 DNA Blood kit)

1.1. Purpose:

This protocol provides a manual method for extracting DNA from dried blood spots (DBS) on filter paper when used in conjunction with Qiagen QIAamp®96 DNA Blood Kit.

1.2. Materials and equipment

- ❖ Qiagen QIAamp®96 DNA mini Kit which contain
 - ✓ ATL, AL, Proteinase K, AW1, and AW2 washing buffer solution
 - ✓ Spin column tube and 2ml collection tube
- ❖ Ethanol (96 – 100%)
- ❖ DBS puncher (for cutting strips of filter paper with DBS)
- ❖ 70% Ethanol
- ❖ Incubator at different temperature (85°C, 56°C, and 70°C)
- ❖ Centrifuge, Test tube rack, 1.5ml microcentrifuge tube
- ❖ Calibrated Micropipets (20 µL, 50µL, 500µL & 1000 µL)
- ❖ Refrigerator (-20°C)

1.3. Safety and Precautions

- ❖ Use proper lab etiquette when working in the laboratory. Wear gloves, lab coat and safety glasses when handling specimens.
- ❖ Use appropriate safety precautions when using DBS puncher. Wipe the cutting tool with 70% alcohol between samples.
- ❖ Ensure that all equipment (pipettes, centrifuge, incubator, etc.) is properly calibrated prior to use.
- ❖ Take utmost care during pipetting steps to prevent cross contamination of samples.
- ❖ Ensure that Buffer AW1 and Buffer AW2 have been prepared
- ❖ Store extracted DNA at -20 °C
- ❖ Check the bottles of ATL and AL buffers. If a precipitate has formed, dissolve by incubating at 56 °C before use.
- ❖ All centrifugation steps are carried out at room temperature (15–25°C).

1.4.Procedures

1. Place 3 punched-out circles from a dried blood spot into a 1.5 ml microcentrifuge tube and add 180 μ l of Buffer ATL.
 - ❖ Cut 3 mm diameter punches from a dried blood spot with a single-hole paper puncher.
2. Incubate at 85°C for 10 min. briefly centrifuge to remove drops from inside the lid.
3. Add 20 μ l proteinase K stock solution. Mix by vortexing, and incubate at 56°C for 1 hr. briefly centrifuge to remove drops from inside the lid.
 - ❖ Note: The addition of proteinase K is essential.
4. Add 200 μ l Buffer AL to the sample. Mix thoroughly by vortexing, and incubate at 70°C for 10 min. briefly centrifuge to remove drops from inside the lid. To ensure efficient lysis, it is essential that the sample and Buffer AL are mixed immediately and thoroughly.
 - ❖ Note: Do not add proteinase K directly to Buffer AL. A white precipitate may form when Buffer AL is added to the sample. In most cases, the precipitate will dissolve during incubation. The precipitate does not interfere with the QIAamp procedure or with any subsequent application.
5. Add 200 μ l ethanol (96–100%) to the sample, and mix thoroughly by vortexing. Briefly centrifuge to remove drops from inside the lid.
 - ❖ It is essential that the sample and ethanol are mixed thoroughly.
6. Carefully apply the mixture from step 5 to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 $\times g$ (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate. Close each QIAamp Mini spin column to avoid aerosol formation during centrifugation.
7. Carefully open the QIAamp Mini spin column and add 500 μ l Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000 $\times g$ (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate.
8. Carefully open the QIAamp Mini spin column and add 500 μ l Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 $\times g$; 14,000 rpm) for 3 min.

9. Recommended: Place the QIAamp Mini spin column in a new 2 ml collection tube(not provided) and discard the old collection tube with the filtrate. Centrifuge at fullspeed for 1 min.

❖ This step helps to eliminate the chance of possible Buffer AW2 carryover.

10. Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube (notprovided), and discard the collection tube containing the filtrate. Carefully open theQIAamp Mini spin column and add 150 µl Buffer AE or distilled water. Incubateat room temperature (15–25°C) for 1min, and then centrifuge at 6000 x g (8000 rpm)for 1 min.

11. Storing the extracts at -20°C.

2. Protocol for DNA Amplification using PCR

2.1. Preparation of PCR working solution for SNP 376 & 202

PCR for SNP 376 AND 202	1 x (25µl)	100x (1700 µl)
Promega master mix (Taq in)	12.5 µl	1250 µl
Primer 376F, 10µm	0.25 µl	25 µl
Primer 376R, 10µm	0.25 µl	25 µl
Nuclease –free Water	4 µl	400µl
DNA template	8 µl	

DNA amount based on Ethiopia 2011 survey samples

2.2. SNP 376 & SNP 202 PCR reaction conditions

Initial denaturation	94°C	5 min	1 cycle
Denaturation	94°C	45 sec	32 cycles
Annealing	64°C	30 sec	
Extension	72°C	45 sec	
Final extension	72°C	5 min	1 cycle
Hold	4°C	∞	

2.3. Preparation of PCR working solution for SNP 563

PCR for SNP 563	1 x (25µl)	100x (1700 µl)
Promega master mix (Taq in)	12.5 µl	1250 µl
Primer 563F2, 10µm	0.25 µl	25 µl
Primer 563R2, 10µm	0.25 µl	25 µl
Nuclease –free Water	4 µl	400µl
DNA template	8 µl	

2.4. SNP 563 reaction conditions

Initial denaturation	94°C	5 min	1 cycle
Denaturation	94°C	1 min	32 cycles
Annealing	57°C	1 min	
Extension	72°C	1 min	
Final extension	72°C	5 min	1 cycle
Hold	4°C	∞	

NOTE: DNA volume increased to have better bands

3. Protocol for RFLP method

Protocol for the RFLP method for the three variants are the same. The only difference is the restricted enzyme used for each variants. RFLP with enzyme FokI for SNP 376, enzyme NlaIII for SNP 202, and enzyme MboII for SNP 563.

3.1. Working solution preparation for RFLP

Master mix volume		10µl
	Nuclease free water	7.5µl
	Cutsmart 10x buffer	2µl
	FokI/NlaIII/MboII	0.5 µl
PCR product (template) volume		10µl
Total reaction volume		20 µl

After mixing the PCR template with the master mix, incubate at 37°C for 60 minutes and inactivating at 65°C for 25 minutes.

3.2. Agarose gel solid medium (2%) preparation procedures

1. Measuring 2 grams of agarose powder using electronic digital balance
2. Measuring 100 milliliter of 1X Tris-borate-EthyleneDiamine Tetra Acetic acid (EDTA) buffer (TBE buffer) using graduated cylinder.
3. Mixing and stirring the Agarose powder with the TBE buffer
4. Heating the mixed solution in Microwave oven until completely dissolved (usually three minutes).
5. Adding 10 milliliters of nucleic acid stain to the melted solution (gel) for nucleic acid visualization
6. Cooling the mixture to 60°C and pouring into the casting tray for solidification.
7. Placing the comb in its appropriate position
8. Immediately removing the comb after the gel is solidified
9. Keeping the gel in its plastic and pouring the TBE buffer until to fully immerse the gel during electrophoresis
10. After mixing 5 µl PCR product with 2 µl loading dye, loading the mixed products in the wells.
11. As nucleic acids are negatively charged, wells should be placed towards the negative electrode.
12. Connecting the electrode with power source and adjusting the power at 80 volts.

Final running 2% gel and check the gel on UVP and record the bands in the lab book

Annex 7. EMIS sampling design

A.1 Introduction

For the 2015 Ethiopia National Malaria Indicator Survey (EMIS 2015), the third such survey conducted in Ethiopia, a nationally representative sample of 13,875 households from 555 enumeration areas (EAs) was selected. The survey was designed to provide information on the following key malaria control indicators: (1) the proportion of households having at least one long-lasting insecticide-treated net (LLIN), (2) indoor residual spraying (IRS) status of households, (3) the proportion of study participants who slept under LLIN the night before the survey, (4) the proportion of children under five years of age who slept under an LLIN the night before the survey, and (5) the proportion of pregnant women who slept under an LLIN the night before the survey. The survey produced representative estimates for the main EMIS indicators for household populations in malarious areas of the country as a whole, for urban and rural areas separately, for each region's malarious areas, and for areas between 2,000m and 2,500m above sea level (ASL). The administrative structure of Ethiopia is regional state, zone, woreda (i.e., district), and kebele (i.e., village).

A.2 Sample frame

The sampling frame, a complete list of all eligible enumeration areas (EAs) that entirely cover a given domain, allows a probability selection of sampling units. The sampling frame used for this survey was the most recently available list of enumeration areas (the primary sampling units) and digitized EA maps from the CSA. All EAs were stratified into three strata. Stratum I contained EAs with a mean altitude of $\leq 2,000$ m ASL, Stratum II contained EAs with a mean altitude of $>2,000$ and $\leq 2,500$ m ASL, and Stratum III contained EAs with mean altitude of $>2,500$ m ASL. If an EA fell within two strata, it was classified based on whichever altitude class made up 75 percent of its area. EAs falling in Stratum III were excluded from the sampling frame. The list of eligible EAs was thoroughly evaluated by CSA and the EMIS technical working group before it was used. Each EA had a size measurement (population and/or number of households) and in each domain a sample of EAs with the predetermined sample size was then selected independently with probability proportional to this measure of size. Maps of the selected EAs were used to clearly define boundaries.

The design for the survey was cross-sectional, with a representative probability sampling technique to produce national and sub-national estimates for malarious areas of the country as a whole and stratified for urban/rural development (in the lower stratum [i.e., <2,000m]). The following domains of estimation were specified for the 2015 EMIS:

- National (country): rural for enumeration area (EA) mean altitude of $\leq 2,000$ m ASL.
- National (country): urban for EA mean altitude of $\leq 2,000$ m ASL.
- National (country): for EA mean altitude of $> 2,000$ and $\leq 2,500$ m ASL.
- Sub-national for EA mean altitude of $\leq 2,000$ m ASL: Tigray, Afar, Amhara, Oromia, Somali, Benishangul-Gumuz, SNNPR, Gambella, Harari, and Dire Dawa.

Single population proportion formula was used to determine the minimum sample size required for the survey per domain.

$$n = \frac{Z_{\alpha}^2 P(1 - P)}{d^2} (DEFT)^2$$

Where:

n=the required minimum sample size

P=expected proportion

d=absolute precession= $2 * P * RSE$

RSE=Relative Standard Error (relative precession) of the expected proportion

DEFT=Design effect for accounting for the two-stage cluster sampling method

The key indicator that was taken to calculate the samples size was proportion of children under five years of age who slept under a mosquito net the previous night from the 2011 EMIS. With the proportion of children under five who slept under a mosquito net the previous night being 38.2 percent (2011 EMIS), a relative precession of 9 percent, 95 percent CI, design effect of 2, 72 percent of households with children under five (estimated/calculated from EMIS 2011), and a 20 percent non-response rate, n=13,880 households, and EAs=555.

Based on the above formula, the sample size calculated was 799. Adjusting for the 20 percent non-response rate gives 999 ($=799/0.80$) under-five children per region. The number of households needed depends on the average number of children under five per household. Using the estimated 72 percent of households with under-five children, the number of households required per domain was 1,388 ($=999/0.72$). Since the 2015 EMIS was supposed to produce regional level estimates, the total sample size for the country was the sample size obtained above multiplied by the number of malarious regions, 13,880 ($=1,388 \times 10$).

The survey was primarily designed to provide estimates for areas below 2,000m for each malarious region and national-level estimates for areas with a mean altitude of $>2,000$ and $\leq 2,500$ m ASL, since the upper altitude stratum of $\geq 2,000$ m to $\leq 2,500$ m is affected by infrequent epidemics and, hence, is an area of minor concentration of malaria control activities by the NMCP and FMOH. Accordingly, 85 percent of the HHs were allocated to malarious areas $<2,000$ m ASL and 15 percent to areas $\geq 2,000$ m to $\leq 2,500$ m ASL. To have regional-level estimates of some of the indicators, the number of EAs at regional level should be adequate with acceptable relative precision and design effect. During distribution of the total country-level sample size to the regions, the size of the population living in malaria risk areas ($E < 2,000$ m) was considered. Since precisions were considered at the regional level, a power allocation with an appropriate power value was used to guarantee sufficient sample size in small regions. A condition was imposed such that the sample size in a given region should not be smaller than a given value. For this purpose, the stratum sample (power) allocation section of the RBM guidelines for sampling was used. Power value of 0.22 was appropriate for the condition imposed that the sample size should not be smaller than 647 households in each region.

Table A1. Number of households in the sampling frame*Distribution of the households in the sampling frame by region and residence, Ethiopia 2015*

Region	Number of households in frame			
	Malarious areas ≤2,000m ASL			Areas>2,000m and ≤2,500m ASL
	Urban	Rural	Total	
Tigray	100,503	336,113	436,616	241,818
Afar	44,866	180,253	225,119	0
Amhara	206,041	1,007,195	1,213,236	831,027
Oromia	470,233	2,365,189	2,835,422	1,248,367
Somali	43,904	123,878	167,782	0
Benshangul Gumuz	27,500	124,471	151,971	0
SNNPR	233,434	1,497,911	1,731,345	618,834
Gambela	18,424	37,936	56,360	0
Harari	28,552	11,637	40,189	3,217
Dire Dawa	54,505	22,113	76,618	0
Total	1,227,962	5,706,696	6,934,658	2,943,263

A.3 Sampling design and implementation

Sampling for the 2015 EMIS was a two-stage stratified cluster sampling from the sampling frame. Stratification was achieved by separating the country into three strata by altitude as malarious areas below 2,000m ASL and areas between 2,000m and 2,500m ASL. Malarious areas below 2,000m ASL were further stratified into urban and rural and by region.

In total, 14 sampling strata were created. Samples were selected independently in each sampling stratum, by a two-stage selection process. In the first stage, 555 enumeration areas were selected

with a stratified probability proportional to size selection, according to the sample allocation given in Table A2. After the first stage of selection, and before the main survey, a household listing operation was carried out in all of the selected villages.

The household listing operation consisted of visiting each of the 555 selected EAs, recording the name of each household head, and recording the GPS coordinates of each household. The resulting list of households served as the sampling frame for the selection of households in the second stage.

At the second stage, 25 households were selected from the new household listing for each selected village. Household selection was performed in the field by team leaders using EpiSample.

Table A2 shows the sample allocation of clusters and households by region and by residence. The sample allocation was population proportional to size (PPS): 555 EAs were selected; 466 EAs from malarious areas and 89 from areas $>2,000\text{m}$ and $\leq 2,500\text{m}$ ASL.

Table A2. Sampling allocation of clusters and households*Sample allocation of clusters and households by region, according to residence, Ethiopia 2015*

Region	Malarious areas $\leq 2,000\text{m ASL}$						Areas $>2,000\text{m}$ and $\leq 2,500\text{m ASL}$					
	Allocation of enumeration areas			Allocation of households			Allocation of enumeration areas			Allocation of households		
	Urban	Rural	Total	Urban	Rural	Total	Urban	Rural	Total	Urban	Rural	Total
Tigray	12	37	49	295	06	1,201	5	11	16	120	273	393
Afar	9	34	43	221	23	1,044	0	0	0	0	0	0
Amhara	10	51	61	237	1,249	1,486	3	19	22	73	465	538
Oromia	12	65	77	295	,617	1,912	4	16	20	100	399	499
Somali	10	30	40	212	673	885	0	0	0	0	0	0
Benshangul Gumuz	7	32	39	168	768	936	0	0	0	0	0	0
SNNPR	9	54	63	204	1,310	1,514	2	21	23	46	511	557
Gambela	11	21	32	263	479	742	0	0	0	0	0	0
Harari	20	9	29	495	219	14	1	6	7	25	148	173
Dire Dawa	24	9	33	549	192	741	0	1	1	0	19	19
Total	124	342	466	2,939	8,236	11,175	15	74	89	364	1,815	2,179

Table A3 shows the distribution of the expected number of completed individual interviews with women ages 15–49 by urban and rural residence for each region in malarious areas and for areas $>2,000\text{m}$ and $\leq 2,500\text{m ASL}$.

Table A3. Sample allocation of completed interviews with women

Sample allocation of expected number of completed interviews with women and men by region, according to residence, Ethiopia 2015

Region	Malarious areas ≤2,000m ASL			Areas >2,000m and ≤2,500m ASL
	Women 15–49			
	Urban	Rural	Total	
Tigray	292	766	1,058	297
Afar	210	751	961	0
Amhara	225	1,077	1,302	500
Oromia	296	1,443	1,739	446
Somali	190	503	693	0
Benishangul Gumuz	158	605	763	0
SNNPR	160	1,122	1,282	442
Gambela	254	418	672	0
Harari	416	201	617	151
Dire Dawa	431	142	573	16
Total	2,632	7,028	9,660	1,852

A4. Sample probabilities and sample weights

Because of the non-proportional allocation of the sample to the different reporting domains, sampling weights were required for any analysis using EMIS 2015 data to ensure the sample was representative. Because the EMIS 2015 sample was a two-stage stratified cluster sample, sampling weights were calculated based on sampling probabilities that were calculated separately for each sampling stage and for each cluster.

The following notations were used:

P_{1hi} : sampling probability of the i th cluster in stratum h

P_{2hi} : sampling probability within the i th cluster for households

P_{hi} : overall sampling probability of any households of the i th cluster in stratum h

Let a_h be the number of EAs selected in stratum h , M_{hi} the number of households according to the sampling frame in the i^{th} EA, and $\sum M_{hi}$ the total number of households in the stratum. The probability of selecting the i^{th} EA in the EMIS 2015 sample is calculated as follows:

$$\frac{a_h M_{hi}}{\sum M_{hi}}$$

Let b_{hi} be the proportion of households in the selected cluster compared to the total number of households in EA i in stratum h if the EA is segmented, otherwise $b_{hi} = 1$. Then the probability of selecting cluster i in the sample is:

$$P_{1hi} = \frac{a_h M_{hi}}{\sum M_{hi}} \times b_{hi}$$

Let L_{hi} be the number of households listed in the household listing operation in cluster i in stratum h , let g_{hi} be the number of households selected in the cluster. The second stage's selection probability for each household in the cluster is calculated as follows:

$$P_{2hi} = \frac{g_{hi}}{L_{hi}}$$

The overall selection probability of each household in cluster i of stratum h is therefore the production of the two stage's selection probabilities:

$$P_{hi} = P_{1hi} \times P_{2hi}$$

The sampling weight for each household in cluster i of stratum h is the inverse of its overall selection probability:

$$W_{hi} = 1/P_{hi}$$

The design weight is also adjusted for household non-response and individual non-response to get the sampling weights for households and for women, respectively. Nonresponse is adjusted at the sampling stratum level. For the household sampling weight, the household design weight is multiplied by the inverse of the household response rate, by stratum. For the women's individual sampling weight, the household sampling weight is multiplied by the inverse of the women's individual response rate, by stratum. After adjusting for nonresponse, the sampling weights are normalized to get the final standard weights that appear in the data files. The normalization process is done to obtain a total number of un-weighted cases equal to the total number of weighted cases at the national level, for the total number of households and women. Normalization is done by multiplying the sampling weight by the estimated sampling fraction obtained from the survey for the household weight and the individual woman's weight. The normalized weights are relative weights, which are valid for estimating means, proportions, ratios, and rates, but not valid for estimating population totals or for pooled data.

Annex 8. Study proposal ethical clearance

ADDIS ABABA UNIVERSITY
COLLEGE OF HEALTH SCIENCES
SCHOOL OF PUBLIC HEALTH



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ጤና ሳይንስ ኮሌጅ
የህብረተሰብ ጤና ት/ቤት

Ref. No: SPH/ 285 /09
Date: November 18, 2016

To: Dr. Yimtubezinash W/Ammanuel
Chair, Institutional Review Board
College of Health Sciences

From: Wakgari Deressa (PhD)
Dean and Associate Professor
School of Public Health



Subject: Ethical Clearance and Approval of Research Proposal

Dear Dr. Yimtubezinash,

The Research and Ethics Committee (REC) of the School of Public Health (SPH) has reviewed and approved the following PhD research proposal by **Ashenafi Assefa** (PI):

- ***Malaria elimination in Ethiopia: Advanced tools in molecular epidemiology (Primary Advisor: Prof Ahmed Ali)***

The Academic Commission of the School on its 12th extra-ordinary meeting held on 18 November 2016 approved this research proposal. This is, therefore, to kindly request your good office to review and approve the aforementioned research proposal for implementation. Enclosed please find the minutes of the School AC and REC, and the hard and electronic copies of the proposal.

With Kind Regards!

Encl.

- 12th Extra-Ordinary SPH AC Minutes (2 pages)
- Minutes of the SPH REC
- Hard and electronic copies of the proposal

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Annex 9. Material Transfer Agreement (MTA)

Material Transfer Agreement

Ministry of Science and Technology, Federal Democratic Republic of Ethiopia

National Health Research Ethics Review Committee

Address: Tel.: +251 114 674353 P.O. Box: 2490 Fax: +251114 660241

e-mail: nrerc2002@gmail.com

Addis Ababa –Ethiopia

This Material Transfer Agreement (MTA) has been prepared for use by Ethiopian Public Health Institute (EPHI) and United States Centers for Disease Control & Prevention (CDC), Atlanta, Georgia, USA in all transfer of research material (samples, derivatives, and specimens) related to the protocol,

Malaria Elimination in Ethiopia: Relevance of Advanced Molecular/Diagnostic Tools in Epidemiological Studies

Provider: Ethiopian Public Health Institute, Addis Ababa, Ethiopia

Recipient: Centres for Disease Control & Prevention (CDC), Atlanta, Georgia, USA

1. Provider agrees to transfer to recipient's designated (Dr. Venkatachalam Udhayakumar) the following research materials (specimen).

- ***A dried blood spots will be obtained from Malaria Indicator Survey , for the following tests***
 - G6PD RFLP (G6PD mutation detection)
 - Serology study
 - Parasite detection
 - Unspecified related studies

The research material will only be used for research purposes as described in the protocol by recipient's investigator in designated laboratory for the research project described below, under suitable containment conditions. This research material will not be used for commercial purposes such as screening, production or sale for which a commercialization license may be required. Recipient agrees to comply with all National and International guidelines rules and regulations applicable to the Research Project and the handling of the Research Material.

a) Are the Research materials of human origin?

Yes ☒ No ☐

b) If yes, are they collected according to the details in the protocol and in adherence to National Health Research Ethics Review Committee (NERC) recommendations and their approval

Yes ☒ No ☐

2. This research material and its derivatives will be used by recipient's investigator solely in connection with the following research project

Malaria Elimination in Ethiopia with specificity as follows

- ***A dried blood spots will be obtained on enrolment and day of each subsequent recurrence, for the following tests***
 - G6PD RFLP (G6PD mutation detection)
 - Serology study
 - Parasite detection
 - Unspecified related studies

3. In all presentations or written publications concerning the research project, recipient will seek agreement of provider and acknowledge provider's contribution of this research material unless requested otherwise.

4. This research material represents a significant contribution on the part of provider and is considered proprietary to provider. Recipient therefore agrees to retain control over this research Material and further agrees not to transfer the research Material to other people not under her/his direct supervision without advance written approval of provider. The research material will be disposed of as agreed upon per protocol at the end of the project which is five years from the completion of specimen collection expected by August 2022.

5. The provider does not take any responsibility for loss, damage, wastage or spoilage of the research material during or after shipment to the address provided by the Recipient under conditions agreed to in the protocol of shipment of the samples. This Research Material is provided as a service to the research community. IT IS BEING SUPPLIED TO RECIPIENT WITH NO WARRANTIES, EXPRESS OR IMPLIED, INCLUDING ANY WARRANTY OF MERCHANTABILITY OR FITNESS FOR A

PARTICULAR PURPOSE. Provider makes no representations that the use of the research material will not infringe any patent or proprietary right of third parties.

6. The recipient shall notify the provider in writing of any intention, improvement, modification discovery or development to the material or the information made by Recipient or parties, collaborating with Recipient, here in after referred to as “invention”. Nothing in this agreement shall, however, be construed as conveying to the provider any rights under any patents or other intellectual property to such invention, other than as explicitly provided herein. At its option the provider shall be entitled to receive sample of any materials derived from the Materials for its own research and evaluation purposes only.

7. The under- signed provider and Recipient expressly certify and affirm that the contents of any statements made herein are truthful and accurate.

8. Any additional terms (use an attached page if necessary):

9. The provider maintains, ownership right of the research material and its derivatives unless stated otherwise. The provider will retain a copy (aliquot) of every sample sent abroad as much as possible for local research needs.

Material Transfer Agreement Signature page

For Recipient:

Recipient’s Investigator

Duly Authorized

Signature

Signature/ Stamp

Date -----

Date -----

Mailing Address for Material:

Mailing Address for

Notices:

Tel:-----
Fax:-----

Tel:-----
Fax:-----

For Provider

Provider's Investigator

Duly Authorized

Signature

Signature/ Stamp

Date -----

Date -----

Mailing Address for Material:

Mailing Address for

Notices:

P.O.Box -----

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Annex 11. Statement of originality

We, the PhD candidate and the candidate's Principal Supervisor, certify that the following text, figures and diagrams are the candidate's original work.

- All text, pictures, diagrams and tables unless and otherwise specified are original works of the PI
- All the laboratory data were collected by the PI at the Centers for Disease Control in Atlanta and at the Ethiopian Public Health Institute (EPHI), except the PCR data used for the sub-patent infection analysis that was collected by Dr. Brain Walkman at the CDC.
- All data summary and analysis were made by the PI with the support and supervision from advisors and experts at the CDC, LSHTM and Tulane Universities
- Malaria Indicator surveys (MISs) 2011 and 2015 were planned and implemented by the EPHI in collaboration with the MIS steering committee. The PI of this work was the lead coordinator of the two surveys representing the Ethiopian Public Health Institute (EPHI).
- Bayesian Krgging maps were produced in collaboration with experts at Mahidol oxford Research Unit (MORU), Thailand
- All manuscripts were drafted and submitted by the PI

Name of Candidate: Ashenafi Assefa

Name of Principal supervisor: Ahmed Ali (Prof.)

Annex 10. Conference presentations and recognitions

1. World malaria day conference, May 6, 2019. Axum Ethiopia.
2. 30th Ethiopian Public Health Association Annual conference, February 25-27, 2018. Adama, Ethiopia.
3. 4th Ethiopian Public Health Institute science congress, May 6-8, 2019. Addis Ababa, Ethiopia
4. 65th Annual conference of American society of Tropical Medicine and Hygiene (ASTMH). November 13-17, 2016. Atlanta, USA
5. 67th Annual conference of American society of Tropical Medicine and Hygiene (ASTMH). October 28 – November 1, 2018. New Orleans, USA
6. 14th International Conference on Parasitology (ICOPA), August 19-24, 2018. Daegu, South Korea.
7. 11th Malaria Research Network Symposium, December 17-18, 2019. Debre-Markos, Ethiopia.

Annex 12. Original Papers

Original Papers

Paperr I: Multiplex serology demonstrate cumulativeprevalence and spatial distribution of malariain Ethiopia

Paper I

RESEARCH

Open Access



Multiplex serology demonstrate cumulative prevalence and spatial distribution of malaria in Ethiopia

Ashenafi Assefa^{1,2*} , Ahmed Ali Ahmed², Wakgari Deressa², Heven Sime¹, Hussein Mohammed¹, Amha Kebede², Hiwot Solomon⁴, Hiwot Teka², Kevin Gurrata⁶, Brian Matel⁶, Brian Wakeman⁶, G. Glenn Wilson⁷, Ipsita Sinha^{8,9}, Richard J. Maude^{8,9,10}, Ruth Ashton¹¹, Jackie Cook¹², Ya Ping Shi⁶, Chris Drakeley¹², Lorenz von Seidlein^{8,9}, Eric Rogler⁶ and Jimmie Hwang¹³

Abstract

Background: Measures of malaria burden using microscopy and rapid diagnostic tests (RDTs) in cross-sectional household surveys may incompletely describe the burden of malaria in low-transmission settings. This study describes the pattern of malaria transmission in Ethiopia using serological antibody estimates derived from a nationwide household survey completed in 2015.

Methods: Dried blood spot (DBS) samples were collected during the Ethiopian Malaria Indicator Survey in 2015 from malarious areas across Ethiopia. Samples were analysed using bead-based multiplex assays for IgG antibodies for six *Plasmodium* antigens: four human malaria species-specific merozoite surface protein-1 19kD antigens (MSP-1) and Apical Membrane Antigen-1 (AMA-1) for *Plasmodium falciparum* and *Plasmodium vivax*. Seroprevalence was estimated by age, elevation and region. The seroconversion rate was estimated using a reversible catalytic model fitted with maximum likelihood methods.

Results: Of the 10,278 DBS samples available, 93.6% (9622/10,278) had valid serological results. The mean age of participants was 15.8 years and 53.3% were female. National seroprevalence for antibodies to *P. falciparum* was 32.1% (95% confidence interval (CI) 29.8–34.4) and 25.0% (95% CI 22.7–27.3) to *P. vivax*. Estimated seroprevalences for *Plasmodium malariae* and *Plasmodium ovale* were 8.6% (95% CI 7.6–9.7) and 3.1% (95% CI 2.5–3.8), respectively. For *P. falciparum* seroprevalence estimates were significantly higher at lower elevations (< 2000 m) compared to higher (2000–2500 m) (aOR 4.4; $p < 0.01$). Among regions, *P. falciparum* seroprevalence ranged from 11.0% (95% CI 8.8–13.7) in Somali to 65.0% (95% CI 58.0–71.4) in Gambela Region and for *P. vivax* from 4.0% (95% CI 2.6–6.2) in Somali to 36.7% (95% CI 30.0–44.1) in Amhara Region. Models fitted to measure seroconversion rates showed variation nationally and by elevation, region, antigen type, and within species.

Conclusion: Using multiplex serology assays, this study explored the cumulative malaria burden and regional dynamics of the four human malaras in Ethiopia. High malaria burden was observed in the northwest compared to the east. High transmission in the Gambela and Benishangul-Gumuz Regions and the neglected presence of *P. malariae* and *P. ovale* may require programmatic attention. The use of a multiplex assay for antibody detection in low transmission settings has the potential to act as a more sensitive biomarker.

Keywords: Multiplex serology, Seroprevalence, Malaria, Ethiopia

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Background

Malaria transmission in Ethiopia is non-stable, heterogeneous and seasonal, with peak periods following the main rains from June to September and short rains from March to April [1]. Historically, malaria epidemics occurred at 5 to 8-year intervals. The last malaria epidemic in 2004/2005 caused two million clinical cases and claimed approximately 3000 lives [2, 3]. Although malaria transmission has been declining over the past 15 years [4, 5], 60% of the Ethiopian landmass is suitable for malaria transmission and communities remain vulnerable for malaria transmission. The National Malaria Control Programme of Ethiopia considers areas below 2000 m elevation as malaria endemic and eligible for malaria control activities [6]. *Plasmodium falciparum* and *Plasmodium vivax* are the major documented malaria parasite species, with approximately 1.2 million *P. falciparum* cases and 678,000 *P. vivax* cases reported in 2015 [7]. The proportion of *P. vivax* cases has been increasing whereas a decline in *P. falciparum* incidence was observed [8].

With the decline in malaria cases, measuring malaria transmission intensity and dynamics is becoming increasingly challenging. Transmission estimates using standard malaria diagnostic tests, such as microscopy or rapid diagnostic tests (RDT), entomological inoculation rate, and splenomegaly prevalence fail to detect changes in the malaria burden in very low transmission settings [9]. The last three Ethiopian Malaria Indicator Surveys (EMIS) reported very low prevalence, using microscopy and RDTs: 0.9% by microscopy in 2007, 1.3% by microscopy and 4.5% by RDTs in 2011, and 0.5% by microscopy and 1.2% by RDT in 2015 [10–12]. With decreasing parasite prevalence as well as density of individual infections, the sensitivity of conventional diagnostic tools has been declining [13, 14]. Serological and molecular epidemiological studies may be more useful in such scenarios, and are being increasingly used for measuring malaria burden in low transmission settings [15–17]. Robust seroprevalence estimates of *Plasmodium* infections could be critical for monitoring and evaluation of ongoing malaria control and elimination activities in Ethiopia.

In this study, multiplex serological methods were used to estimate malaria exposure, transmission patterns, and spatial and regional distribution of malaria in Ethiopia, using samples collected during the 2015 EMIS.

Methods

Study area

The study was conducted as part of the National Ethiopian Malaria Indicator Survey in 2015 (EMIS-2015). Ethiopia has successfully implemented three national EMISs in 2007, 2011 and 2015 [10–12]. EMIS-2015 was embarked during the peak of malaria transmission

season, between September and December 2015. The sampling design provided national and sub-national estimates of major malaria intervention indicators, including malaria prevalence for malarious areas of the country, as well as estimates for areas below 2000 m elevation, areas between 2000 and 2500 m, and for 10 administrative regions.

Study design and sample collection

EMIS-2015 was a cross-sectional survey that used a two-stage cluster sampling approach. A total of 555 enumeration areas (EAs) were selected proportional to the population size of the regions, as estimated by the Ethiopian Central Statistics Agency. In each EA, 25 households were randomly selected following onsite EA household mapping. A total of 53,335 individuals were surveyed in 13,875 selected households. Demographic, socio-economic, use of malaria prevention, and malariometric data were collected in the selected households from each EA. Children under 5 years of age in each selected household and persons of all ages in every fourth household were eligible for biological sample testing. Whole blood from a finger prick from consenting individuals was collected for a malaria RDT (histidine-rich protein 2 (HRP2) and *Plasmodium* lactate dehydrogenase (pLDH) antigen CareStart®, AccessBio, US), a malaria blood slide (thick and thin blood film), haemoglobin (Hemocue Hb 201+, Hemocue AB, Ängelholm, Sweden) measurement, and dried blood spot (DBS) samples. Whatman 903 Protein Saver (GE Healthcare, Pittsburgh, PA, USA) filter paper was used for DBS collection. Filter papers were air dried, individually packed in a plastic bag together with a desiccant and stored at -20°C at the Ethiopian Public Health Institute (EPHI) before they were shipped for further laboratory-based testing at the US Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA.

Ethical consideration

The EMIS-2015 protocol received ethical clearance from the National Research Ethics Review Committee of Ethiopia and PATH. The survey protocol underwent human subjects review at CDC and was considered to be a non-research programme evaluation activity. Additional ethical clearance for the present serology study was obtained from the Institutional Review Board of the College of Health Sciences of the Addis Ababa University (AAUMF 03-008).

Blood spot elution, bead coupling and antigens used in the study

Laboratory analysis was conducted as previously described [15]. Briefly, a 6-mm punch was used from each DBS sample and blood eluted to a 1:20 concentration

in blocking buffer (Buffer B: Phosphate Buffered Saline (PBS) containing 0.5% Bovine Serum Albumin (BSA), 0.05% Tween 20, 0.02% sodium azide, 0.5% polyvinyl alcohol, 0.8% polyvinylpyrrolidone and 0.5% w/v *Escherichia coli* extract) overnight.

Six *Plasmodium* antigens were used for this study: two *P. falciparum* antigens (Merozoite Surface Protein-1 19kD (MSP-1) and Apical Merozoite Antigen-1 (AMA-1), two *P. vivax* antigens (PvMSP-1 and PvAMA-1), one *P. malariae* (PmMSP-1) and one *P. ovale* (PoMSP-1). The four *Plasmodium* MSP-1 19kD antigens were produced as recombinant proteins and purified as described previously [18]. The external domain of *P. falciparum* AMA-1 antigen was produced at the Walter Reed Army Institute of Research (WRAIR) under previously published conditions [19]. The *P. vivax* AMA-1 antigen was produced at the London School of Hygiene and Tropical Medicine (LSHTM) under previously published conditions [20]. The *Schistosoma japonicum* glutathione-S-transferase antigen (GST) was produced recombinantly and served as a generic protein to assess immunoglobulin G (IgG) non-specific binding.

All antigens were coupled to magnetic beads (Luminex Corp, Austin, TX, USA) as per prior studies [18, 21]. Briefly, beads were pulse vortexed, transferred to a microcentrifuge tube and centrifuged for 1.5 min at 13,000g. Supernatant was removed, and the beads were washed with a 0.1 M and pH 6.2 sodium phosphate (NaP) solution. Beads were activated by suspending in NaP with 5 mg/mL of EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) and 5 mg/mL sulfo-NHS (sulfo N-hydroxysuccinimide) and incubating with rotation for 20 min at room temperature (RT), while protected from light. After a wash with coupling buffer (50 mM 2-(4-morpholino)-ethane sulfonic acid, 0.85% NaCl at pH 5.0), antigens were coupled to beads in the presence of a coupling buffer for 2 h at a concentration of 20 µg/mL for all antigens, except for PvAMA1 and GST at 15 µg/mL. Beads were washed once with PBS and suspended in PBS with 1% bovine serum albumin (BSA) with incubation for 30 min at RT by rotation. Beads were then resuspended in a storage buffer (PBS, 1% BSA, 0.02% sodium azide and 0.05% Tween-20) and stored at 4 °C. Coupled beads were run with a panel of known malaria seronegatives to assure minimal non-specific MFI signal would be given by beads used in this study.

The DBS elution was assayed for IgG antibodies using bead-based multiplex technology, and all wash steps were performed with a handheld magnet. In 5 mL of reagent buffer (Buffer A: PBS, 0.5% BSA, 0.05% Tween-20, 0.02% NaN₃), a bead mix was prepared with all coupled bead regions included (approximately 625 beads/antigen per well), and 50 µL bead mix was pipetted into each well of

a BioPlex Pro plate (BioRad, Hercules, CA, USA). Beads were washed twice with 100 µL PBS with Tween (PBST), and 50 µL of the reagent mix [in 5 mL Buffer A: 1:500 anti-human IgG (Southern Biotech), 1:625 anti-human IgG₁ (Southern Biotech), 1:200 streptavidin-PE (Invitrogen)] was added to each well. Negative control samples and a dilution curve of hyperimmune serum were added to each assay plate to monitor any change in control values over the course of the study. Samples of blood eluted from DBS were added to predefined wells (already containing beads and reagent mix) at 1:50 dilution. Plates were incubated overnight with gentle shaking at RT and protected from light. The next morning (approximately 16 h total incubation time), plates were washed three times, and beads were re-suspended with 100 µL PBS and read on a MAGPIX machine (Luminex Corp, Austin, TX, USA). Mean fluorescence intensity (MFI) signal was generated for a minimum of 50 beads/region, and the background (bg) MFI from wells incubated with Buffer B was subtracted from each sample to give a final value of MFI-bg. Minimal variation was seen in the assay signal for the background or positive hyperimmune serum curve values, so no plates were re-run for the study.

Statistical analysis

Data analysis was done using Stata 13 software (College Station, USA). Samples with GST MFI-bg reads above 1000 MFI value (non-specific binding) were excluded from the analysis (n = 678). To dichotomize seropositivity, log₁₀-transformed MFI-bg values were fitted to a two-component Finite Mixture Model (FMM) by the FMM procedure with normal distribution and maximum likelihood estimation outputs. A seropositivity cutoff value was determined by the mean MFI-bg value of the first (assumed seronegative) component plus three standard deviations [18]. Overall, *P. falciparum* and *P. vivax* seropositivity were defined as an individual being positive for either or both of the MSP-1 and AMA-1 antigens for each species. Seropositivity estimates for *P. malariae* and *P. ovale* are presented using the seropositivity cut-off method outlined above. However, to minimize cross-reactivity between the species-specific MSP-1 antigens, the current study report an additional conservative two-stage approach that increase specificity, but decrease sensitivity of the specific antibodies to the targeted antigens. For this conservative approach for *P. malariae* and *P. ovale*, individual readings for PmMSP-1 or PoMSP-1 response first had to be above the MFI-bg cutoff as described above. Additionally, the PmMSP-1 and/or PoMSP-1 MFI-bg signal for that sample also needed to be above the PmMSP-1 MFI-bg signal for the same sample (ratio to PmMSP-1 greater than 1.0) to be considered PmMSP-1 and/or PoMSP-1 positive.

A reversible catalytic model was fitted to the dichotomized data using maximum likelihood methods to generate a seroconversion rate (SCR or λ) and a seroreversion rate (ρ). The model was used to generate age seroprevalence curves, from which a seroconversion rate (SCR) representing the force of infection for the community was calculated. Evidence for two forces of infection was investigated, if visual inspection of SCR curves indicated such a comparison and was guided by a profile likelihood plots to determine the most likely time (year) of change in transmission [16, 22]. Multiple logistic regression models were employed to determine odds ratios (OR); 95% confidence intervals (CI) for gender, age and elevations. Similar regression models were employed for *P. falciparum* and *P. vivax* seropositivity. Adjustments were made by region, elevation and age group.

Empirical bayesian kriging in ArcGIS version 10.5 (ESRI, CA, USA) was used to predict the spatial distribution of seroprevalence as a continuous surface of probability of being above a cut-off. This is an established method that uses a statistical model to predict and interpolate the spatial distribution of a variable from available data [23]. Maps were developed separately for MSP-1 and AMA-1 antigens for both *P. falciparum* and *P. vivax* for all individuals and for children under 5 years of age. Seroprevalence by woreda/district was compared with the 2015 annual parasite incidence (API) data. QGIS version 2.1.8 and ArcGIS version 10.5 (ESRI, Redlands, CA) were used to produce maps.

Sampling weights calculated during MIS 2015 were used to ensure the representativeness of the samples tested to the study population.

Results

Study population

Of the expected 15,829 DBS samples, 10,278 individual DBSs were available for the current study, serological results were generated for 93.6% (9622/10,278) of these (Fig. 1). A total of 8944 results were merged with the EMIS-2015 data base, of which 52.3% (95% CI (50.8–53.7)) of the survey participants were female (Table 1). Figure 2 shows the spatial distribution of EAs providing serology data. The overall proportion of samples collected from children under 5 years was 44.8% (95% CI 43.0–46.5); although this proportion was above 60% in the Afar and Somali Regions (Table 1). The number of individuals with serology data within an EA ranged from 1 to 55, with a mean of 24 individuals per EA. Overall mean age was 15.8 years (95% CI 15.2–16.4) (range 1–107). Of the samples collected, 75.0% (95% CI 67.5–81.2) were from areas with elevation less than 2000 m.

A total of 0.8% (95% CI 0.6–1.2) of the participants were positive by RDT; comprising 0.5% (95% CI 0.3–0.7) for *P. falciparum* (HRP2 antigen positive), 0.2% (95% CI 0.1–0.4) for either *P. vivax*, *P. malariae* or *P. ovale* (Pv/Pm/Po) (pan-LDH antigen positive), and 0.1% (95% CI 0.1–0.3) considered as a mixed infection (HRP2 and pan-LDH positive). More than 90% of the positive *P. falciparum* RDT results were from the Gambela and

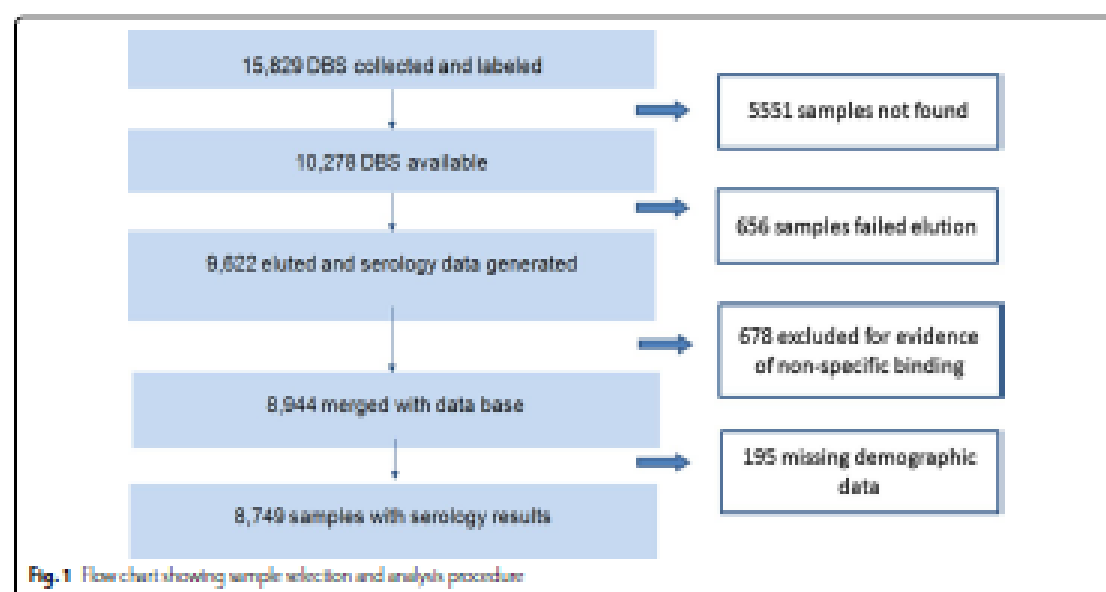
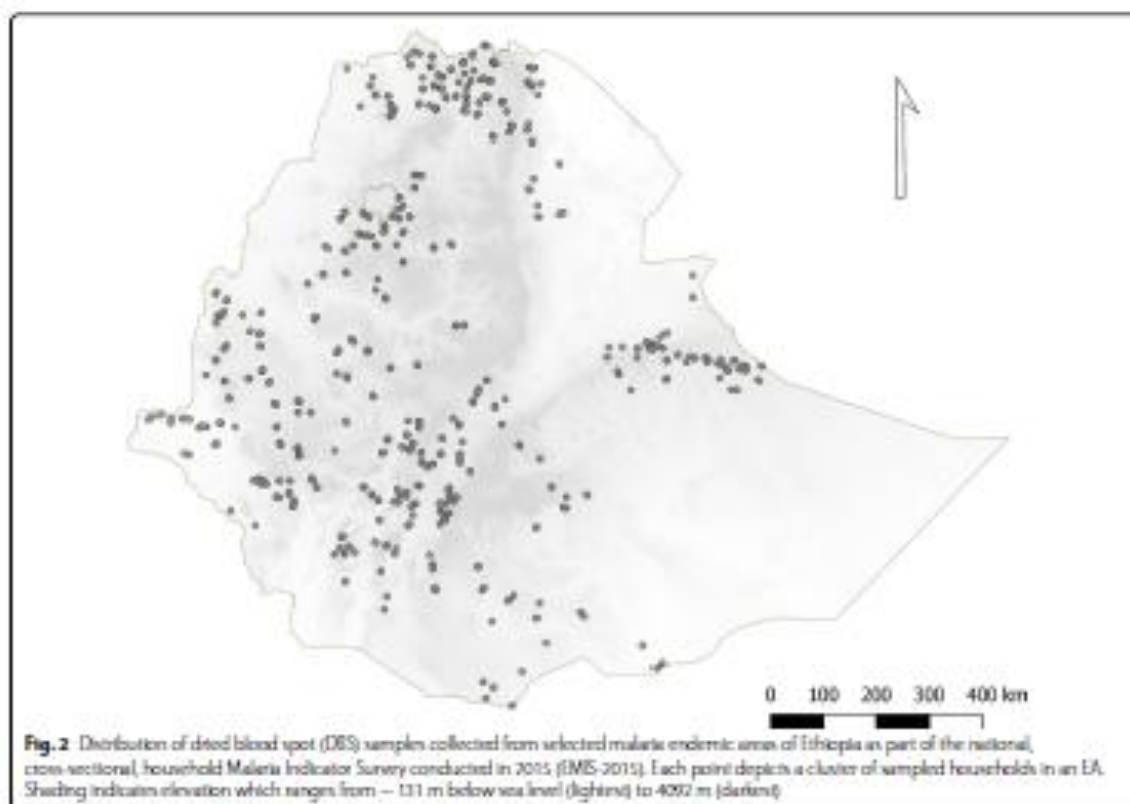


Table 1 Characteristics of study participants by administrative region, Ethiopian Malaria Indicator Survey 2015 (EMIS-2015)

	Tigray	Afar	Amhara	Oromia	Somali	Benishangul-Gumuz	Southern Nations and Nationalities Peoples'	Gambella	Harari	Dire Dawa	National
Gender % (95% CI)											n = 6740
Male	46.5 (44.1–48.9)	47.6 (45.2–50.0)	50.9 (47.4–54.3)	48.2 (45.6–50.8)	45.1 (40.8–49.4)	45.3 (40.8–49.8)	46.4 (44.3–48.5)	41.4 (37.4–45.5)	47.4 (33.0–61.8)	48.5 (38.1–58.9)	47.7 (46.3–49.2)
Female	53.5 (51.1–55.9)	52.4 (50.0–54.8)	49.1 (45.7–52.6)	51.8 (49.1–54.4)	54.9 (50.6–59.2)	54.7 (50.1–59.2)	53.6 (51.5–55.7)	58.6 (54.5–62.6)	52.6 (34.6–70.6)	51.5 (41.6–61.4)	52.3 (50.8–53.7)
Age group (years) % (95% CI)											
0–5	43.8 (39.6–48.0)	64.6 (63.2–66.0)	40.8 (37.3–44.3)	44.6 (41.5–47.7)	61.7 (58.8–64.6)	49.5 (46.5–52.5)	46.4 (43.2–49.7)	54.8 (50.8–58.8)	25.5 (12.9–48.2)	46.6 (35.3–57.9)	44.8 (43.0–46.5)
5–15	17.7 (15.2–20.4)	12.8 (8.2–19.5)	20.5 (18.0–23.1)	18.8 (15.8–21.8)	14.6 (11.2–18.1)	16.0 (12.6–19.4)	20.8 (18.9–22.8)	18.9 (16.4–21.5)	3.5 (1.0–11.4)	10.2 (6.4–15.4)	19.4 (17.8–21.1)
15–25	12.3 (11.3–13.4)	6.1 (5.9–6.4)	12.6 (9.5–16.0)	11.6 (10.0–13.4)	5.2 (3.6–7.5)	11.5 (7.6–16.0)	11.4 (8.7–13.4)	10.7 (8.3–13.6)	22.1 (17.4–27.5)	14.0 (10.9–17.8)	11.7 (10.7–12.6)
25–50	17.1 (14.9–19.4)	14.1 (12.1–16.4)	19.2 (16.2–22.6)	19.7 (17.5–22.1)	14.6 (10.3–18.4)	14.5 (10.3–19.3)	17.1 (15.6–18.6)	12.3 (8.1–18.0)	36.8 (29.3–46.0)	21.7 (15.9–28.9)	18.5 (17.3–19.6)
50+	6.2 (4.6–10.1)	0.5 (1.1–1.5)	6.9 (5.1–9.1)	5.2 (4.0–7.0)	3.6 (2.3–5.3)	6.6 (5.3–12.5)	5.2 (4.1–6.6)	3.2 (2.3–4.5)	12.1 (7.4–19.2)	8.1 (4.5–14.2)	5.7 (4.9–6.6)
Deviation (yr) % (95% CI)											n = 8740**
< 1000	5.6 (1.6–17.4)	38.0 (33.3–43.4)	0 (0.0–0.0)	0 (0.0–0.0)	28.6 (12.6–52.7)	8.2 (1.3–52.7)	0 (0.0–0.0)	88.4 (72.1–93.8)	0 (0.0–0.0)	0 (0.0–0.0)	1.9 (1.2–2.8)
1000–2000	64.3 (64.8–78.6)	62.0 (59.6–64.3)	53.4 (53.2–72.5)	77.4 (67.3–87.4)	71.4 (47.3–87.4)	91.8 (57.4–98.8)	79.6 (67.3–87.3)	71.5 (46.3–92.5)	100 (100.0–100.0)	99.1 (93.9–99.9)	73.1 (65.6–79.4)
> 2000	30.2 (16.5–48.5)	0 (0.0–0.0)	46.6 (27.5–64.8)	22.6 (13.1–34.1)	0 (0.0–0.0)	0 (0.0–0.0)	21 (12.7–32.7)	0 (0.0–0.0)	0 (0.0–0.0)	0 (0.0–0.0)	25.0 (18.8–32.9)
Bed net use (last night) % (95% CI)											n = 5160**
Yes	57.6 (53.3–64.5)	80.5 (83.2–80.9)	59.1 (48.1–69.4)	69.6 (59.6–76.1)	75.3 (61.2–85.4)	66.1 (54.3–76.2)	55.7 (49.4–61.8)	94.7 (78.0–99.0)	31.3 (12.8–63.3)	25 (14.3–41.6)	80.1 (67.5–86.6)
No	42.4 (35.5–49.7)	19.5 (10.1–34.3)	40.9 (30.6–51.6)	30.4 (21.4–40.4)	24.8 (14.6–38.8)	33.9 (23.8–45.7)	44.3 (38.3–50.6)	5.3 (3.4–24.0)	66.3 (36.7–87.2)	74.4 (58.4–83.7)	19.9 (11.4–32.5)
RDT results % (95% CI)											
PI†	0.6 (0.4–1.6)	0 (0.0–0.0)	0.7 (0.2–1.8)	0.2 (0.1–0.6)	0 (0.0–0.0)	6.2 (2.3–16.3)	0.2 (0.1–0.5)	16.8 (11.8–23.2)	0 (0.0–0.0)	0 (0.0–0.0)	0.5 (0.3–0.7)
Pan	0.7 (0.3–1.3)	0.2 (0.0–1.3)	0 (0.0–0.0)	0.1 (0.0–0.4)	0.1 (0.0–0.8)	0 (0.0–0.0)	0.4 (0.1–1.2)	0.1 (0.0–0.6)	0 (0.0–0.0)	0 (0.0–0.0)	0.2 (0.1–0.4)
PI+Pan	0.1 (0.0–0.6)	0 (0.0–0.0)	0.5 (0.1–1.6)	0 (0.0–0.0)	0 (0.0–0.0)	0.4 (0.0–2.6)	0.2 (0.0–0.6)	1.7 (0.9–3.0)	0 (0.0–0.0)	0 (0.0–0.0)	0.1 (0.1–0.3)
Negative	93.4 (97.4–99.0)	99.8 (98.7–100.0)	99.3 (94.7–99.6)	99.7 (99.3–99.9)	99.9 (99.2–100.0)	93.3 (82.3–97.8)	99.3 (98.3–99.7)	81.3 (74.5–86.8)	100 (100.0–100.0)	100 (100.0–100.0)	98.2 (98.8–99.4)

n: number of observations; RDT, rapid diagnostic test; * 95% CI; bold value is presented based on chi-square test; ** RDT results were not done for 4 individuals; † RDT results were invalid and 3 individuals refused RDT testing



Bentshangul-Gumuz Regions. 62.1% (95% CI 57.5–66.6) of respondents said they used a bed net during the previous night (Table 1).

Seroprevalence

Nationally, 32.1% (95% CI 29.8–34.4) of the participants were seropositive for any IgG against the two *P. falciparum* antigens, and 25.0% (95% CI 22.7–27.34) for *P. vivax*. Only 6.6% (95% CI 5.5–8.0) of participants were seropositive for both *P. falciparum* and *P. vivax*, while 41.1% (95% CI 38.5–43.7) of individuals were positive either for *P. falciparum* or *P. vivax*. Eight point six percent (95% CI 7.6–9.7) and 3.1% (95% CI 2.5–3.8) were seropositive for *P. malariae* and *P. ovale*, respectively (Table 2). By antigen, 23.8% (95% CI 21.4–26.3) were positive against *P. falciparum* MSP-1 and 24.5% (95% CI 22.4–26.7) AMA-1, while for *P. vivax*, 21.2% (95% CI 19.2–23.4) were positive against MSP-1 and 14.4% (95% CI 12.7–16.2) AMA-1.

For children under 5 years of age, 18.0% (95% CI 16.1–20.1) and 14.8% (95% CI 12.7–17.2) were seropositive for *P. falciparum* and *P. vivax*, respectively. Similarly, 33.3% (95% CI 30.4–36.2) of male children were seropositive for

P. falciparum compared to 30.9% (95% CI 28.3–33.6) of the females [aOR: 1.3 (95% CI 1.1–1.4), $p < 0.05$] (Additional file 1). A similar trend was observed for *P. vivax*, where 26.4% (95% CI 23.4–29.7) of males compared to 23.6% (95% CI 21.1–26.3) of females were seropositive [aOR: 1.3 (95% CI 1.1–1.5), $p > 0.05$].

Seroprevalence by age

The DBS sample collection was purposely biased towards children under 5 years of age, with 44.8% (95% CI 43.0–46.5) of the DBS samples having been collected from this age group. Seropositivity increased with age group. This pattern was more pronounced for *P. falciparum* than *P. vivax*, although the difference was significant in both *Plasmodium* species ($p < 0.05$).

Conservative estimate for *Plasmodium malariae* and *Plasmodium ovale*

Table 3 shows of all samples tested, 5.1% (95% CI 4.4–6.0) were seropositive for *P. malariae* and 1.5% (95% CI 1.1–2.0) for *P. ovale* using the conservative approach to define seropositivity. Both species were observed in all the regions, albeit with a variable distribution. The

Table 2 Proportion of individuals (all ages and under 5 years) found to be malaria seropositive by species and by antigen in malaria endemic areas of Ethiopia, 2015

<i>Plasmodium</i> species	Antigens	% seropositive (95% CI) (all ages)	% seropositive (95% CI) (under 5 years)
<i>P. falciparum</i>	MSP-1	23.8 (21.4–26.3)	12.3 (10.6–14.2)
	AMA-1	24.5 (22.4–26.7)	11.3 (9.8–13.0)
	PI positive (MSP-1 and/or AMA-1)	32.1 (29.8–34.4)	18.0 (16.1–20.1)
<i>P. vivax</i>	MSP-1	21.2 (19.2–23.4)	12.3 (10.5–14.4)
	AMA-1	14.4 (12.7–16.2)	6.7 (5.5–8.0)
	Pv positive (MSP-1 and/or AMA-1)	25.0 (22.7–27.3)	14.8 (12.7–17.2)
<i>P. malariae</i> ^a	MSP-1	8.6 (7.6–9.7)	6.1 (5.0–7.3)
<i>P. ovale</i> ^a	MSP-1	3.1 (2.5–3.8)	2.0 (1.4–2.7)
Any <i>Plasmodium</i>	PI (MSP-1/AMA-1) or Pv (MSP-1/AMA-1) or Pm MSP-1 or Po MSP-1	44.5 (41.9–47.2)	30.2 (27.3–33.2)
Either <i>P. falciparum</i> or <i>P. vivax</i>	PI (MSP-1/AMA-1) or Pv (MSP-1/AMA-1)	41.1 (38.5–43.7)	26.7 (24.0–29.5)
Both <i>P. falciparum</i> and <i>P. vivax</i>	PI (MSP-1/AMA-1) and Pv (MSP-1/AMA-1)	6.6 (5.5–8.0)	1.7 (1.2–2.4)

Samples positive for Merozoite Surface Protein-1 (MSP-1) and/or Apical Membrane Antigen-1 (AMA-1) for the respective species were considered positive for that species

PI, *Plasmodium falciparum*; Pm, *Plasmodium malariae*; Po, *Plasmodium ovale*; Pv, *Plasmodium vivax*

^a Normal approach (mean seronegative + 3sd) to defining cut-off level used for *P. malariae* and *P. ovale*

Table 3 Proportion seropositive for less common malaria (*P. malariae* Merozoite Surface Protein-1 (PmMSP-1) and *P. ovale* Merozoite Surface Protein-1 (PoMSP-1)), using the conservative approach to defining seropositive cut-off values, by region and age group, in malaria endemic areas of Ethiopia, 2015

Region	All ages		Under 5 years	
	PmMSP-1	PoMSP-1	PmMSP-1	PoMSP-1
Tigray	1.1 (0.6–2.3)	0.6 (0.3–1.1)	1.1 (0.4–2.9)	0.3 (0.1–1.2)
Afar	1.3 (0.6–3.0)	0.6 (0.1–2.7)	1.8 (0.7–4.2)	0.5 (0.1–4.2)
Amhara	5.0 (3.2–7.6)	2.4 (1.8–3.3)	2.7 (1.3–6.2)	1.0 (0.3–3.1)
Oromia	6.3 (5.0–7.8)	2.0 (1.3–3.0)	5.8 (4.4–7.8)	1.8 (1.0–3.3)
Somali	1.3 (0.6–2.8)	0.1 (0.0–0.8)	1.1 (0.4–2.9)	0
Benishangul-Gumuz	2.1 (1.0–4.4)	0.7 (0.2–2.5)	1.3 (0.4–4.7)	1.5 (0.4–4.9)
Southern Nation and Nationalities Peoples	4.9 (3.7–6.3)	0.6 (0.4–1.2)	4.7 (3.1–7.1)	0.7 (0.3–1.8)
Gambela	0.2 (0.1–1.1)	0.1 (0.0–0.5)	0.3 (0.1–1.2)	0
Haran	4.5 (3.6–26.4)	4.0 (0.3–33.6)	0	0
Dire Dawa	2.4 (0.7–8.0)	0.8 (0.1–5.3)	1.7 (0.3–9.9)	0
Total (%)	5.1 (4.4–6.0)	1.5 (1.1–2.0)	4.6 (3.6–5.7)	1.7 (0.8–1.9)

Pm, *Plasmodium malariae*; Po, *Plasmodium ovale*

proportion of seropositives was slightly lower for both species, accordingly to the conservative estimation method. A similar trend of seropositivity was observed for children under 5 years of age (Table 3).

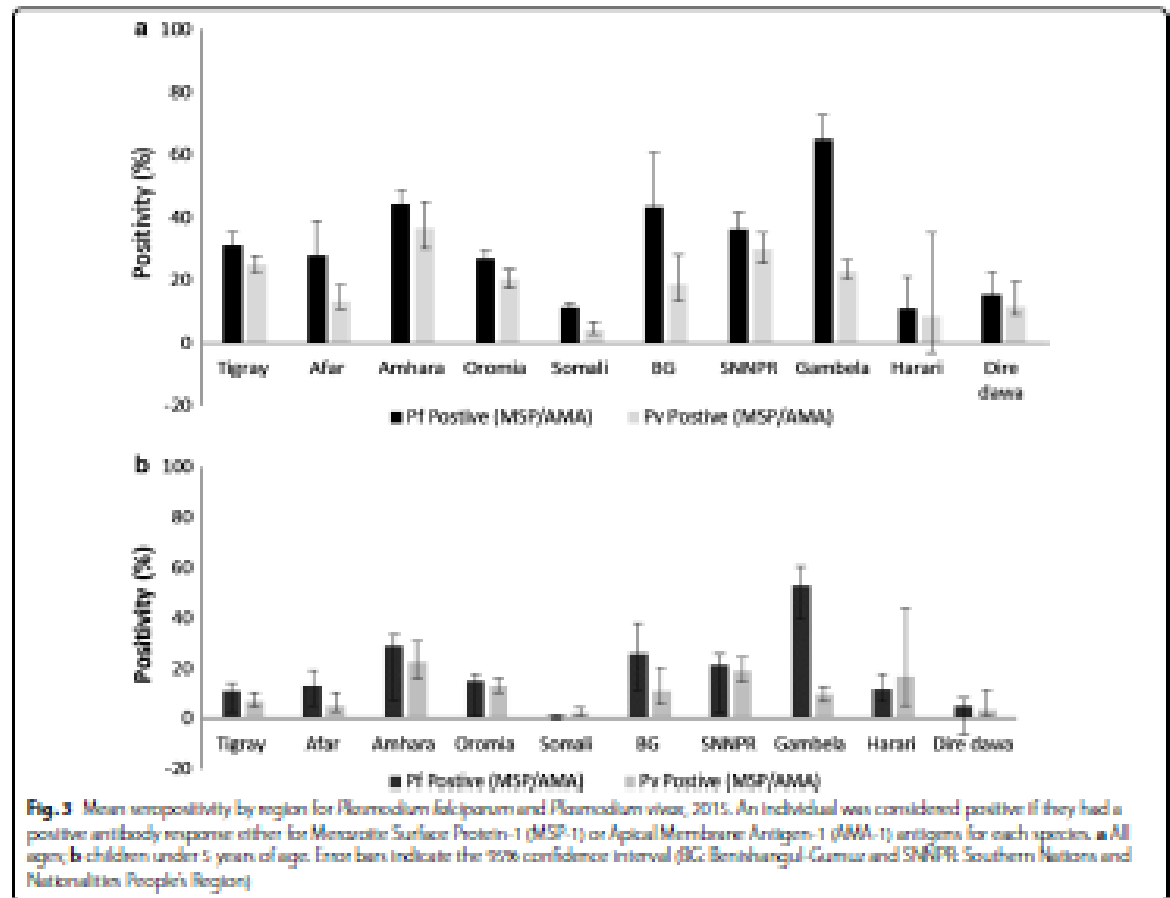
Seroprevalence by region

The proportion of individuals seropositive was variable by region, with *P. falciparum* seropositivity higher than *P. vivax* seropositivity in all the regions (Fig. 3). The proportion seropositive for *P. falciparum* by region ranged from 11.0% (95% CI: 8.8–13.7) in Somali to 65.0% (95% CI: 58.0–71.4) in Gambela Region and for *P. vivax* from 4.0% (95% CI: 2.6–6.2) in Somali to 36.7% (95% CI: 30.0–44.1) in Amhara Region.

The API data from 2014 (API 2014) obtained from the Federal Ministry of Health (Additional file 2), observed high malaria transmission in Gambela, Benishangul-Gumuz and Amhara Regions. By antigen comparison, *P. falciparum* API showed greater association with PfMSP-1 [rho of 0.37 and $p < 0.01$ (spearman correlation)] and *P. vivax* API with PvAMA-1 (rho 0.32 and $p < 0.01$).

Seroprevalence by elevation

Seropositivity was inversely related to elevation for *P. falciparum*, with lower elevation (<2000 m) areas having significantly higher seroprevalence compared to areas of higher elevation (2000–2500 m) [aOR 4.36 (95% CI 2.7–7.0), $p < 0.01$]. The highest seropositivity was observed for *P. falciparum* at elevations below 1500 m and a peak was observed at an elevation below 1000 m (Fig. 4a). A weaker inverse effect of elevation was observed for *P. vivax*. There was no significant difference in the transmission of *P. vivax* between above and below 2000 m (OR 1.5 (95% CI 0.9–2.4), $p > 0.05$). Among children under



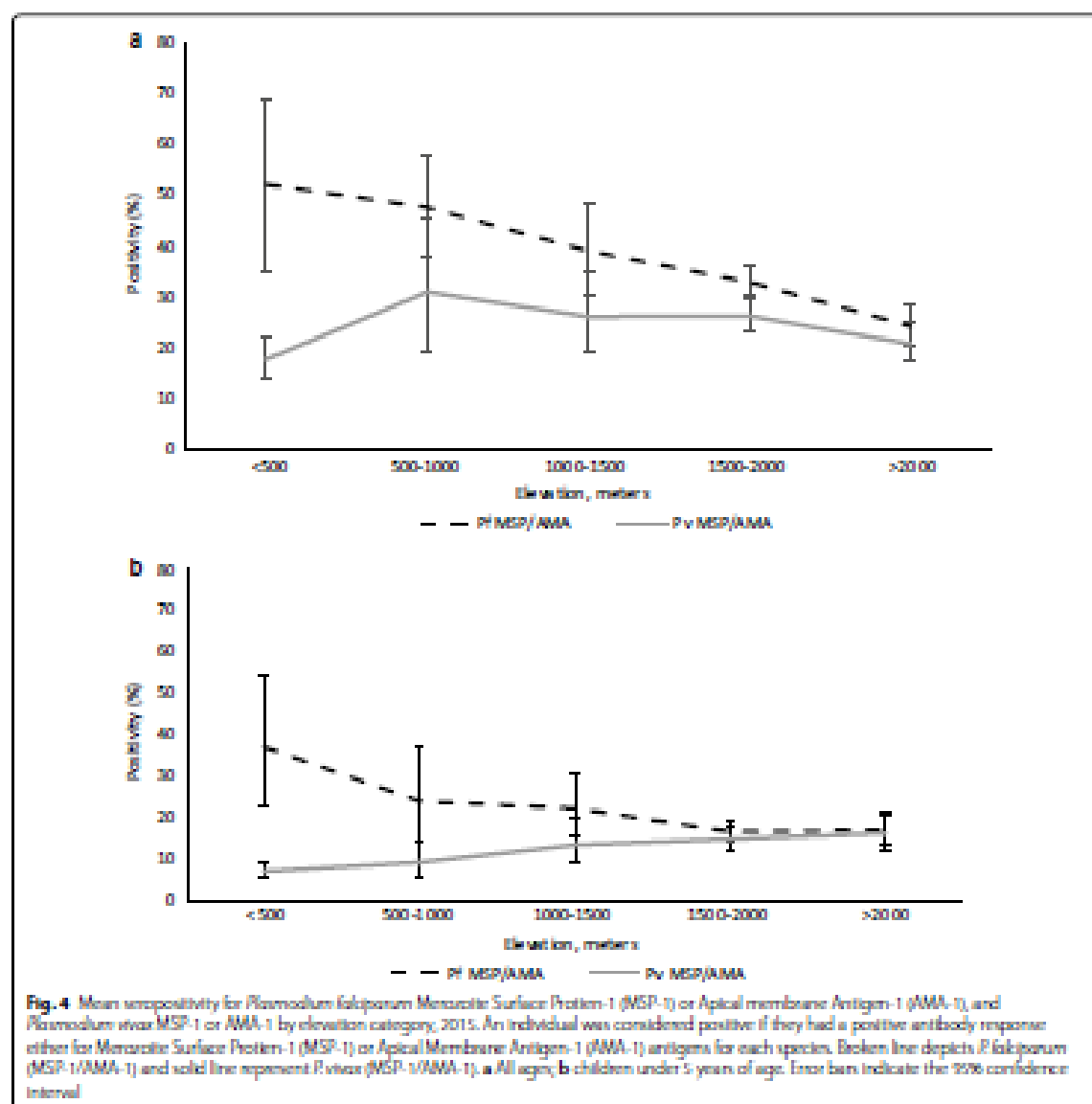
5 years of age, *P. falciparum* and *P. vivax* both showed a significant although varying relationship with elevation. *Plasmodium vivax* showed direct relation with increasing elevation. High *P. vivax* seropositivity was observed at higher elevations above 1500 m (Fig. 4b). No significant effect of elevation on seropositivity was detected for the rare species, *P. malariae* and *P. ovale* ($p > 0.05$).

Generally, seropositivity was found to vary in space, with elevation and among administrative regions, as depicted in Fig. 5. An interpolated surface map predicted differing transmission patterns for *P. falciparum* and *P. vivax*. Higher seropositivity is predicted in the northwest compared to the southeast.

Seroconversion curves

The overall age seroconversion plot for antibody responses for both *P. falciparum* and *P. vivax* are shown in Fig. 6. In general, higher seroconversion rates were observed for *P. falciparum* compared to *P. vivax* both for

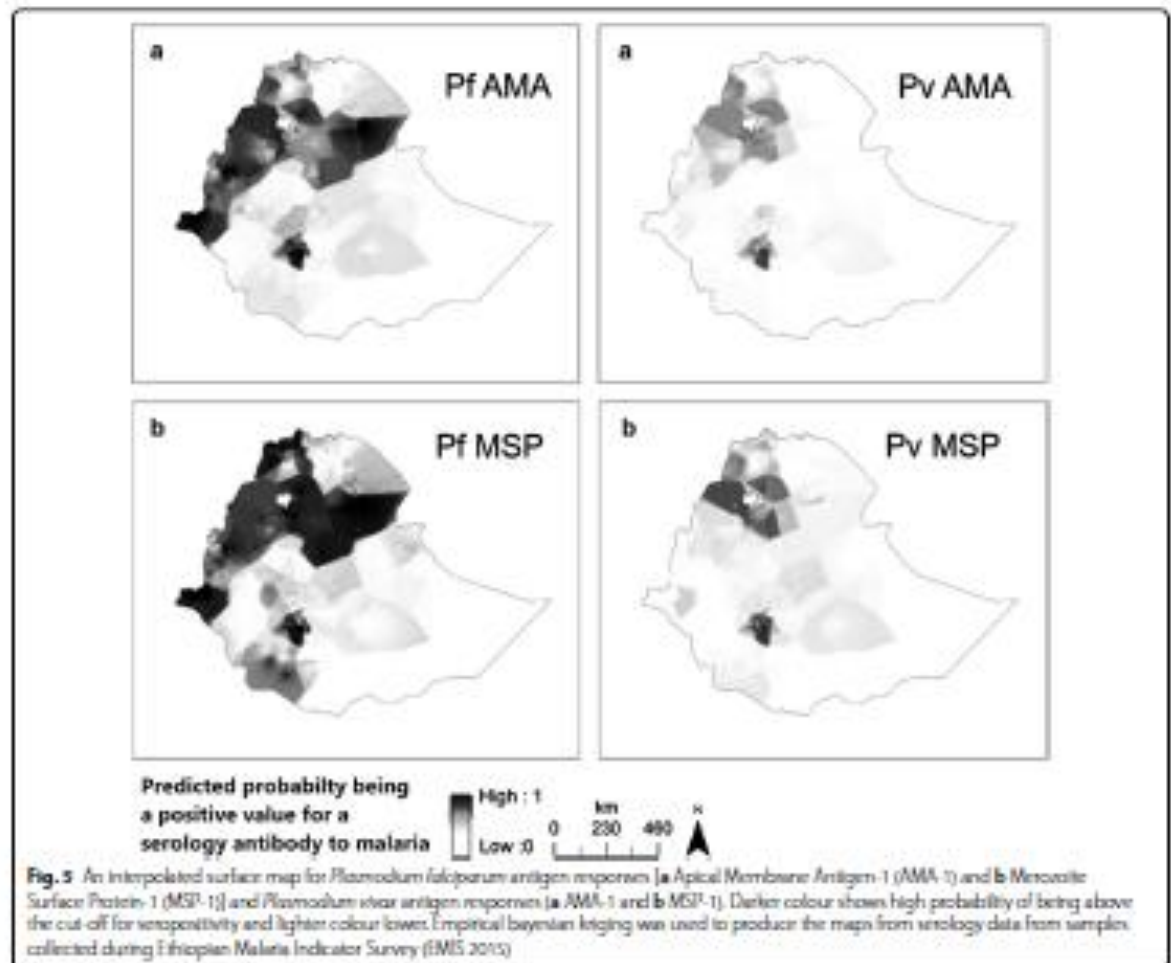
the combined MSP-1/AMA-1 outcome and individual MSP-1 and AMA-1 antigens; and MSP-1 antigen show higher seroconversion rates compared to AMA-1 antigens for both species. Heterogeneity was observed across the different antigen responses. A similar trend was observed when the model was fitted to data with elevation below 2000 m and combined MSP-1 and AMA-1 antigens. Regionally, the seroconversion model was fitted to the four major administrative regions (Amhara, Oromia, SNNPR, Tigray) both for *P. falciparum* and *P. vivax* MSP-1 and AMA-1 antigen responses. Heterogeneous seroconversion rates were observed over the regions (Additional files 3 and 4). For seroconversion plots, where visual inspection indicated better fit between the observed and predicted pattern of transmission, two forces of infection were compared with a single force of infection. This showed a non-significant change over around two decades for both *P. falciparum* and *P. vivax* antigen responses ($p > 0.05$).



Discussion

The current study documented malaria seroprevalence in Ethiopia using samples collected from a national EMIS conducted in 2015. Of all samples analysed, 32.1% had IgG antibodies against combined *P. falciparum* MSP-1 or AMA-1 antigens, and 25.0% for *P. vivax* MSP-1 or AMA-1. The relative numbers were lower for children under 5 years of age at 18.0% for *P. falciparum* and 14.8% for *P. vivax*, suggesting a lower recent transmission in Ethiopia. Seropositivity in children under

5 years of age may accurately represent recent infection as they have had limited exposure and time to generate the cumulative antibody response seen in adults [24–26]. The serological results were cumulative antibody responses for the relatively long-term markers of transmission, MSP-1 and AMA-1 antigens [16, 22, 27–29]. The RDT results for the study samples showed 0.8% positivity for *Plasmodium*, of which 0.5% was due to *P. falciparum*.



Generally, significantly higher levels of *P. falciparum* transmission were observed in areas of lower elevation (<2000 m). This is consistent with previous studies [1, 17, 19], and attributed to the ambient temperature and moisture requirements of the *Anopheles* vector mosquitoes [30]. This reaffirms the national malaria control programme's strategy in which high elevation areas (>2000 m) are considered to be areas where malaria is not transmitted and thus not targeted for vector control but only case management interventions. However, the evidence of increasing *P. vivax* seropositivity with elevation over the range of the study setting could expand the understanding of malaria transmission in Ethiopia. The relapse of *P. vivax* infections may have contributed to the high *P. vivax* prevalence observed at higher elevations [31–35]. Travel history is reported to be a risk factor for malaria exposure in Ethiopia [24–26], but in the

EMIS-2015 only 2% reported travel history a month prior to the survey [12].

Seropositivity was high for children under 5 years of age, particularly in the Gambela and Benishangul Gumuz Regions, although was lower than in adults. These show higher malaria transmission that may need a more intensive approach for malaria control in the affected areas. A larger proportion of males than females were seropositive for both *P. falciparum* and *P. vivax*. Increased malaria risk in men in Ethiopia has been attributed to a greater extent of outdoor, occupational exposure [24–27, 36].

A total of 8.6% of individuals were seropositive for *P. malariae* and 3.1% for *P. ovale*. Variable trends of proportion seropositive were observed in the regions for both species. These findings, albeit with high proportion, support previous reports of sporadic occurrence of *P. malariae* and *P. ovale* that may be responsible for up to

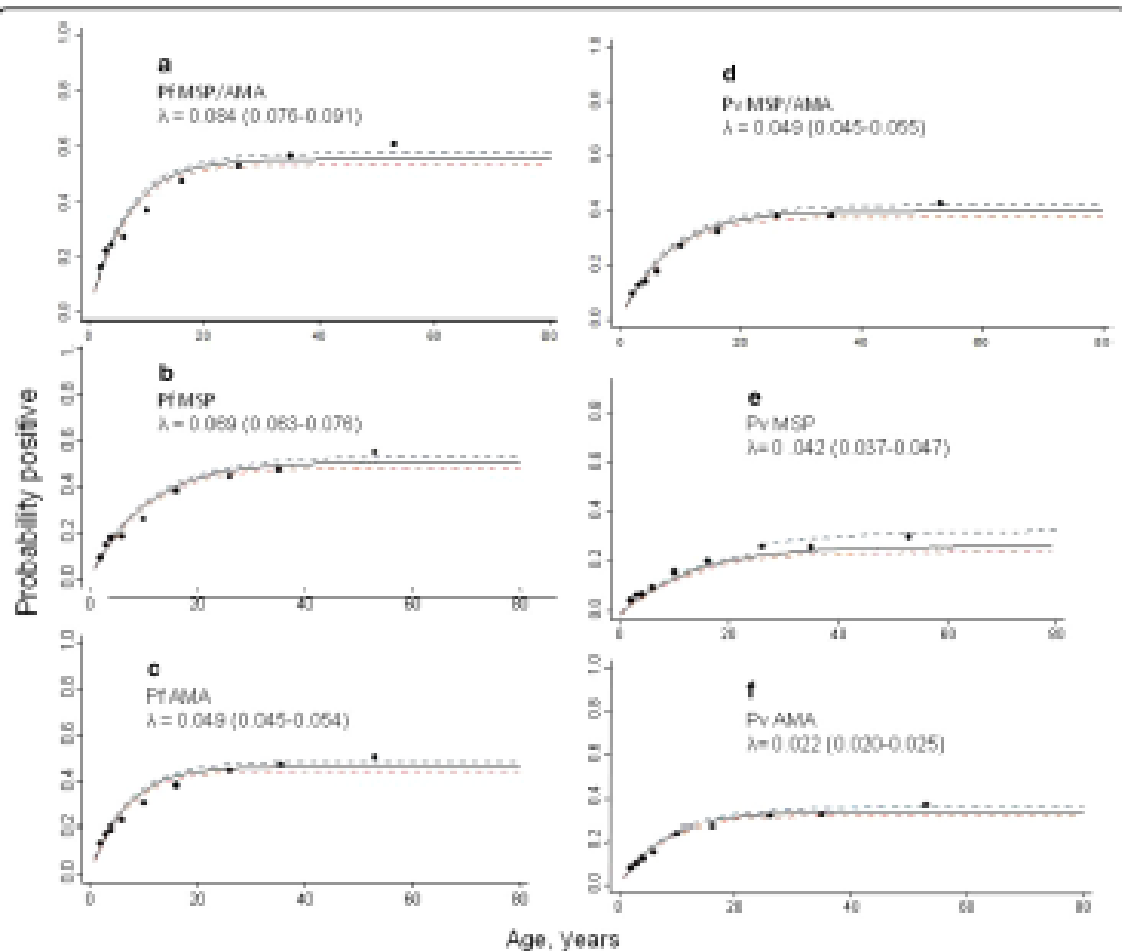


Fig. 6 National age-seroconversion plots for antibody responses for *Plasmodium falciparum* and *Plasmodium vivax* antigens [Merozoite Surface Protein-1 (MSP-1) and Apical Membrane Antigen-1 (AMA-1)]. Y-axis represents probability of being seropositive and the X-axis age. Seroconversion curves represent the rate at which a population becomes seropositive to specific antigens resulting in seroconversion rates (SCR) or lambda (λ). In each graph points represent age seroprevalence (by deciles), unbroken line represents maximum likelihood curves and broken lines represent the 95% confidence interval. a and d depict the seroconversion rates for combined (MSP-1/AMA-1) *P. falciparum* antigen response (a) and for combined (MSP-1/AMA-1) *P. vivax* antigen response (d). Plots b and c show seroconversion rates for individual *P. falciparum* antigens MSP-1 (b) and AMA-1 (c). Plots e and f show seroconversion rates for individual *P. vivax* antigens MSP-1 (e) and AMA-1 (f)

1% of the malaria incident cases in Ethiopia [30, 37–40]. Currently there is no reporting system for these *Plasmodium* species, *P. malariae* and *P. ovale*, in Ethiopia. Antibody cross-binding among the different MSP-1 antigens has been documented as non-substantial [18, 41]. In the present study, data was analysed using an additional conservative approach to defining seropositivity to rule out the possibility of IgG cross-binding, thus reporting the most conservative estimates for comparison. Using this approach would potentially increase specificity but decrease sensitivity. The overall seropositivity, although

reduced, was 5.1% for *P. malariae* and 1.5% for *P. ovale*, with the regional trend and pattern of transmission remaining similar.

The use of serological methods in measuring malaria transmission in low-transmission settings has been documented in a number of studies [15, 16, 27, 36, 42, 43]. Application of serology methods was suggested for Ethiopia because of the very low prevalence of malaria through RDT and microscopy methods across several national surveys [27, 36, 42]. The estimate was too low to track progress over the years or give detailed information

about differences in malaria burden by region or risks of infection due to demographic or behavioural factors. By describing exposure to malaria over a longer period, serology data were able to better document differences in exposure between age groups, regions and by elevation.

The findings show the magnitude of transmission intensity was highly variable among different regions and elevations in Ethiopia. Yalew et al. reported similar trends with 30.0 and 21.8% seroprevalence for *P. falciparum* and *P. vivax*, respectively, for the Amhara region in 2013 [36]. The current study reported 32.1% for *P. falciparum* and 25.0% for *P. vivax* for the same region. The slight variation in seropositivity may be attributed to the different type of laboratory assay used. The challenge of comparing data from ELISA and bead-based multiplex serology has been investigated previously [15]. Results from multiplex serology were more robust, sensitive and ideal for large-scale surveys compared to ELISA [15]. Heterogeneity of response for the different antigens was demonstrated in several studies. Ashton et al. [27] described MSP-1 antigens to be better biomarkers for *P. falciparum*, although Cook et al. [16] reported AMA-1 to be better for measuring *P. falciparum* in low-transmission settings. Characterization of antigen markers may be required to further qualify the serology methods [44]. The current finding was in agreement with the API data from 2014 obtained through routine surveillance. Although routine surveillance can provide crucially important data for decision making for countries at lower transmission, additional biomarkers to track malaria burden can serve as ancillary data as surveillance systems continue to improve and dynamically evolve in completeness and quality.

The study had several limitations. Only 65% of DBS samples from the EMIS-2015 survey were available for serological analysis. Although no systematic bias was introduced and frequencies were weighted, the representativeness of this sample at the national and regional levels should be interpreted cautiously. The data were insufficient to produce refined spatial heat maps and the maps produced show only general trends. Samples collected are assumed to reflect the malaria exposure of their respective locations and did not account for potential population movement. Although the survey reported very low recent travel history, past travel encompassing the previous harvest season as well as previous years were not captured and could reflect past malaria exposure risk from a different locale. This study assessed long-term markers of transmission, but further characterizing antigens based on local eco-epidemiology is recommended to identify and select specific short- and long-term markers.

Conclusion

The current study explored the malaria burden and transmission intensity using serological markers of exposure to four malaria species. It documented the magnitude, spatial distribution and dynamics of transmission over differing elevation, administrative regions, and age groups using samples collected during a national programme evaluation survey with minimal sample preparation time and cost. These findings confirmed the National Malaria Control Programme data of the relative high transmission in the Gambela and Benishangul-Gumuz Regions and highlighted gaps in the current understanding of malaria epidemiology. The increase of *P. vivax* seropositivity with elevation and the presence of *P. malariae* and *P. ovale* in all administrative regions (given *P. ovale* requires hypnozoitocidal treatment) may require programmatic consideration in selecting diagnostic tools and appropriate treatment guidelines. These results can be used as baseline data to evaluate the recent malaria elimination efforts in Ethiopia especially as routine surveillance systems are progressively being strengthened.

Additional files

Additional file 1. Percent malaria seropositivity and Odds Ratio (OR) by sex, age and elevation for each species in malaria endemic areas of Ethiopia, 2014.

Additional file 2. An interpolated surface map for *P. falciparum* (left) and *P. vivax* (right). Empirical Bayesian geiging was used to produce the maps from the Annual Parasite Incidence data in Ethiopia (API 2014).

Additional file 3. Regional age-seroconversion plots for antibody responses for *Plasmodium falciparum* antigens. Y-axis represents probability of being seropositive and the X-axis age. Seroconversion curves represent the rate at which a population become seropositive to specific antigens resulting in seroconversion rates (SCR) or lambda (λ). In each graph points represent age seroprevalence (by deciles), unbroken line represents maximum likelihood curves and broken lines represent the 95% confidence interval. Plots A and B depict the seroconversion curves for *P. falciparum* antigens response to MSP-1 (A) and AMA-1 (B) for the major four regions Tigray, Amhara, Oromia and Southern Nations and Nationalities People's Regions.

Additional file 4. Regional age-seroconversion plots for antibody responses for *P. falciparum* antigens. Y-axis represents probability of being seropositive and the X-axis age. Seroconversion curves represent the rate at which a population become seropositive to specific antigens resulting in seroconversion rates (SCR) or lambda (λ). In each graph points represent age seroprevalence (by deciles), unbroken line represents maximum likelihood curves and broken lines represent the 95% confidence interval. Plots A and B depict the seroconversion curves for *P. vivax* antigens response to MSP-1 (A) and AMA-1 (B) for the major four regions, Tigray, Amhara, Oromia and Southern Nations and Nationalities People's Region (SNNPR).

Abbreviations

AMA-1: Apical Membrane Antigen-1; API: annual parasite incidence; aOR: adjusted odds ratio; BG: Benishangul-Gumuz; BSA: bovine serum albumin; CDC: Center for Disease Control; CI: confidence interval; DBS: dried blood

spot; EA: enumeration assay; ELISA: Enzyme Linked Immunosorbent Assay; EMS: Ethiopia Malaria Indicator Survey; FMM: Finite Mixture Model; GST: Glutathione-S-Transferase; HRP2: Histidin Rich Protein 2; IgG: Immune globulin G; LSHTM: London School of Hygiene and Tropical Medicine; MFI: mean fluorescence intensity; MSP-1: Merozoite Surface Protein-1; NEREC: National Ethics and Research Review Committee; OR: odds ratio; PBS: phosphate buffered saline; PCR: polymerase chain reaction; pLDH: plasmodium lactate dehydrogenase; RDT: rapid diagnostic test; RFLP: restriction fragment length polymorphism; RT: room temperature; SCR: seroconversion rate; SNNPR: Southern Nations and Nationalities People's Region; WRAIR: Walter Reed Army Institute of Research.

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Authors' contributions

Conceived the study: AA, JH, AAA, WD designed the experiments: AA, ER, YP, BW. Performed the experiments: AA, ER, RG, BM. Analyzed the data: AA, ER, JK, CD, RA, RM, ES, JH. Assisted study implementation: AAA, WD, AK, HS, JM, HM, JH. Critically reviewed and contributed to writing: JH, LS, ER, AAA, GW, RM, WD. Drafted the manuscript: AA. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The MIS 2015 protocol received ethical clearance from the National Ethics and Research Review Committee (NEREC) of Ethiopia and from the College of Health Sciences Addis Ababa University. The survey protocol which included serology testing underwent human subject review at CDC and received non-research determination.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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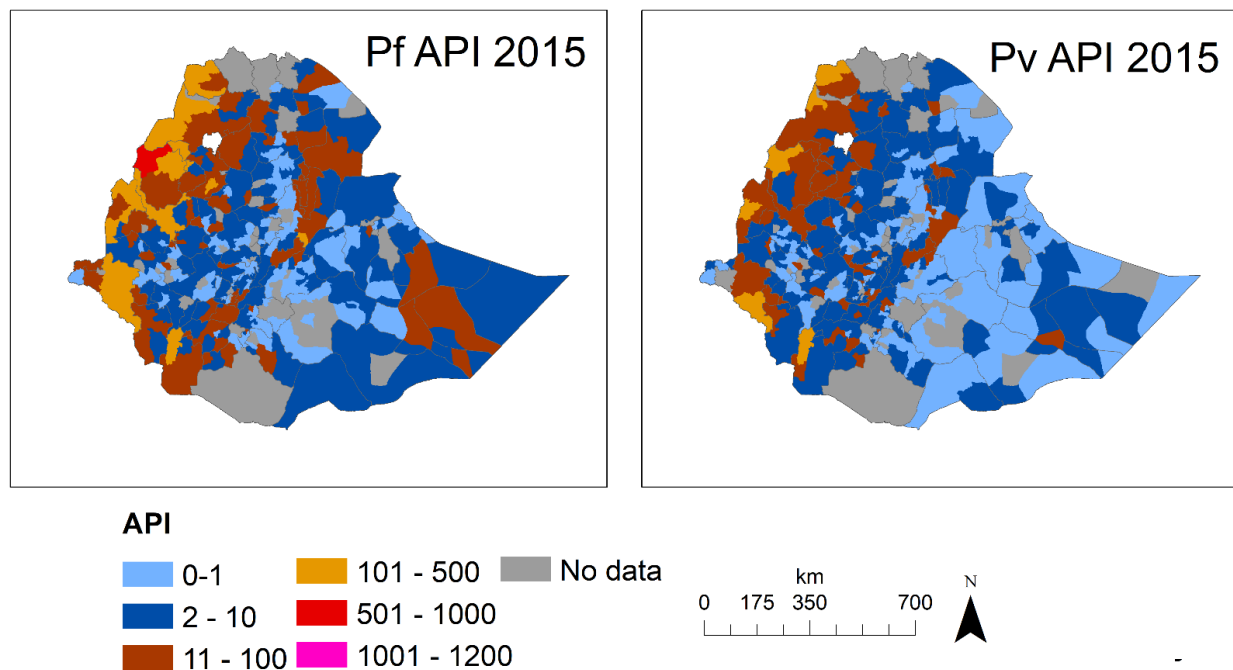


Additional files

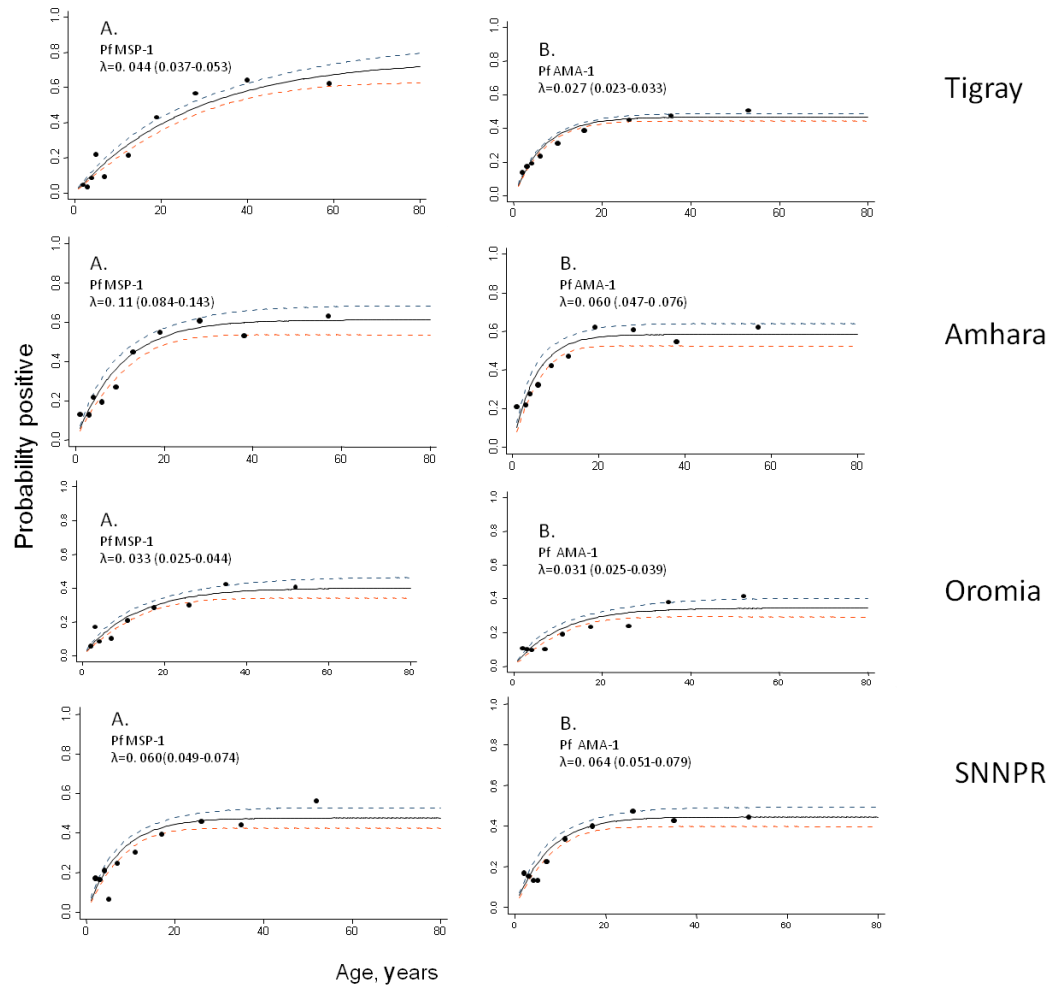
Additional file 1. Percent malaria seropositivity and Odds Ratio (OR) by sex, age and elevation for each species in malaria endemic areas of Ethiopia.

	<i>P. falciparum</i>			<i>P. vivax</i>		
	% Seropositive (MSP-1 or AMA-1)	Unadjusted OR (95% CI)	Adjusted OR (95% CI)‡	% Seropositive (MSP-1 or AMA-1)	Unadjusted OR (95% CI)	Adjusted OR (95% CI)‡
Sex						
Female	30.9 (28.3-33.6)	Ref	Ref	23.6 (21.1-26.3)	Ref	Ref
Male	33.3 (30.4-36.2)	1.1 (1.0-1.2)	1.3 (1.1-1.4)**	26.4 (23.4-29.7)	1.2 (1.0-1.4)	1.3 (1.1-1.5)
Age (Years)						
<5	18.0 (16.0-20.2)	Ref	Ref	14.8 (12.7-17.3)	Ref	Ref
5-15	31.5 (27.6-35.6)	2.2 (1.9-2.5)*	2.3 (2.0-2.6)*	25.7 (22.5-29.2)	2.5 (2.2-2.9)*	2.5 (2.2-3.0)*
15-25	43.7 (38.6-48.8)	3.8 (3.3-4.5) *	4.0 (3.5-4.7)*	34.0 (28.7-39.8)	4.6 (4.0-5.5)*	4.5 (3.8-5.4)*
25-50	50.5 (45.4-55.5)	5.2 (4.6-5.9)*	6.5 (5.6-7.5)*	36.7 (32.7-40.8)	6.6 (5.7-7.6)*	4.9 (4.2-5.7)*
≥50	58.2 (52.0-64.2)	6.3 (5.1-7.8)*	8.0 (6.4-9.9)*	43.7 (36.8-50.8)	8.3 (6.7-10.3)*	6.2 (5.0-7.6)*
Elevation (meters)						
>2000	24.4 (20.6-28.6)	Ref	Ref	20.9 (17.4-24.9)	Ref	Ref
1000-2000	34.3 (31.4-37.3)	1.6 (1.3-2.1)*	1.8 (1.4-2.4)*	26.4 (23.4-29.5)	1.4 (1.0-1.8)	1.5 (1.1-2.0)**
<1000	49.9 (40.3-59.5)	3.1 (2.0-4.8)*	4.4 (2.7-7.0)*	24.6 (17.2-33.9)	1.2 (0.7-2.1)	1.5 (0.9-2.3)

**P-values <0.05; * P-values <0.01 ‡ Controlled for listed risk factors

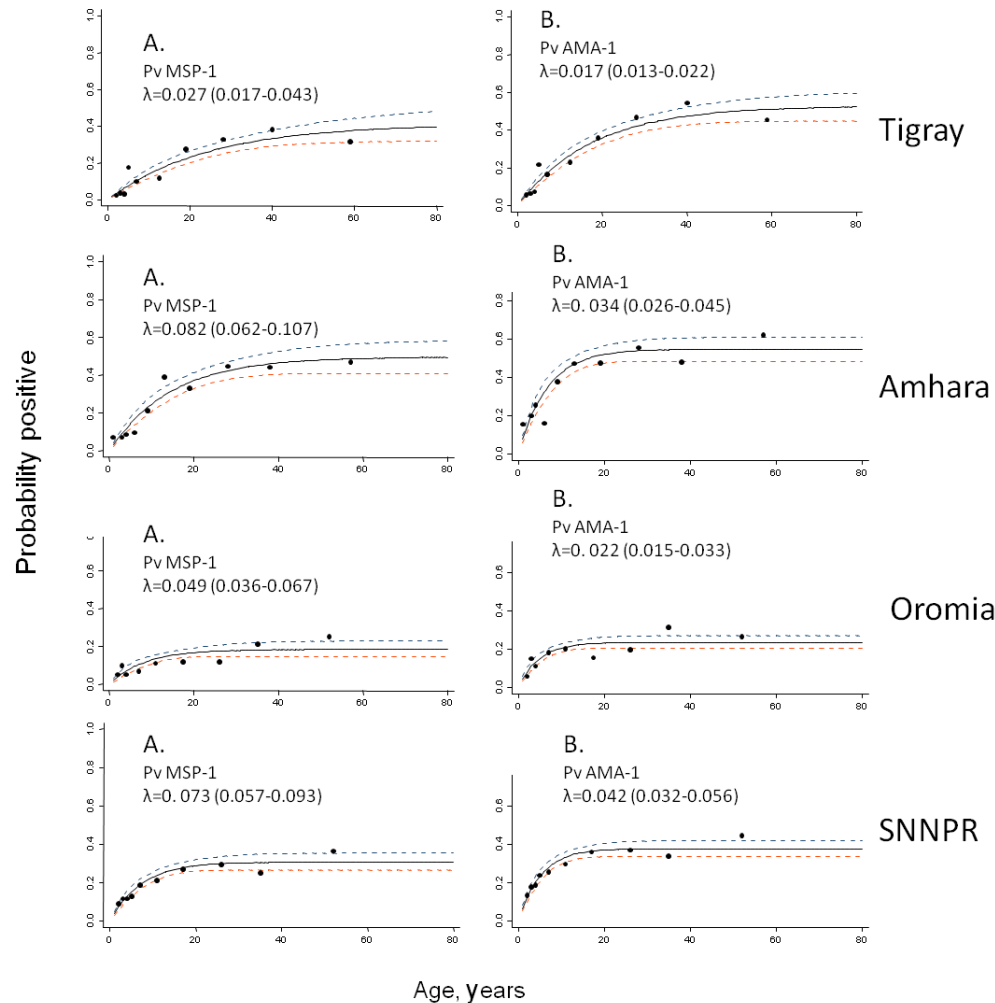


- 1
- 2 Additional file 2. An Interpolated surface map for *P. falciparum* (left) and *P. vivax* (right).
- 3 Empirical bayesian kriging was used to produce the maps from the Annual Parasite Incidence data
- 4 in Ethiopia (API 2014)



5

6 Additional file 3: Regional age-seroconversion plots for antibody responses for *Plasmodium*
7 *falciparum* antigens. Y-axis represents probability of being seropositive and the X-axis age.
8 Seroconversion curves represent the rate at which a population become seropositive to specific
9 antigens resulting in seroconversion rates (SCR) or lambda (λ). In each graph points represent age
10 seroprevalence (by deciles), unbroken line represents maximum likelihood curves and broken lines
11 represent the 95% confidence interval. Plots A and B depict the seroconversion curves for *P.*
12 *falciparum* antigens response to MSP-1 (A) and AMA-1(B) for the major four regions Tigray,
13 Amhara, Oromia and Southern Nations and Nationalities People's Regions.



14

15 Additional file 4: Regional age-seroconversion plots for antibody responses for *P. falciparum*
 16 antigens. Y-axis represents probability of being seropositive and the X-axis age. Seroconversion
 17 curves represent the rate at which a population become seropositive to specific antigens resulting
 18 in seroconversion rates (SCR) or lambda (λ). In each graph points represent age seroprevalence
 19 (by deciles), unbroken line represents maximum likelihood curves and broken lines represent the
 20 95% confidence interval. Plots A and B depict the seroconversion curves for *P. vivax* antigens
 21 response to MSP-1 (A) and AMA-1 (B) for the major four regions, Tigray, Amhara, Oromia and
 22 Southern Nations and Nationalities People's Region (SNNPR).

Paper II. Assesment of subpatent *plasmodium* evaluation of subpatent infection in northwestern Ethiopia

Paper II

RESEARCH

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Assessment of subpatent *Plasmodium* infection in northwestern Ethiopia

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Abstract

Background: Ethiopia has set a goal for malaria elimination by 2030. Low parasite density infections may go undetected by conventional diagnostic methods (microscopy and rapid diagnostic tests) and their contribution to malaria transmission varies by transmission settings. This study quantified the burden of subpatent infections from samples collected from three regions of northwestern Ethiopia.

Methods: Sub-samples of dried blood spots from the Ethiopian Malaria Indicator Survey 2015 (EMIS-2015) were tested and compared using microscopy, rapid diagnostic tests (RDTs), and nested polymerase chain reaction (nPCR) to determine the prevalence of subpatent infection. Paired seroprevalence results previously reported along with gender, age, and elevation of residence were explored as risk factors for *Plasmodium* infection.

Results: Of the 2608 samples collected, the highest positive rate for *Plasmodium* infection was found with nPCR 3.3% (95% CI 2.7–4.1) compared with RDT 2.8% (95% CI 2.2–3.5) and microscopy 1.2% (95% CI 0.8–1.7). Of the nPCR positive cases, *Plasmodium falciparum* accounted for 3.1% (95% CI 2.5–3.8), *Plasmodium vivax* 0.4% (95% CI 0.2–0.7), mixed *P. falciparum* and *P. vivax* 0.1% (95% CI 0.0–0.4), and mixed *P. falciparum* and *Plasmodium malariae* 0.1% (95% CI 0.0–0.3). nPCR detected an additional 30 samples that had not been detected by conventional methods. The majority of the nPCR positive cases (61% (53/87)) were from the Benishangul-Gumuz Region. Malaria seropositivity had significant association with nPCR positivity [adjusted OR 10.0 (95% CI 3.2–29.4), $P < 0.001$].

Conclusions: Using nPCR the detection rate of malaria parasites increased by nearly threefold over rates based on microscopy in samples collected during a national cross-sectional survey in 2015 in Ethiopia. Such subpatent infections might contribute to malaria transmission. In addition to strengthening routine surveillance systems, malaria programmes may need to consider low-density, subpatent infections in order to accelerate malaria elimination efforts.

Keywords: Subpatent infections, Asymptomatic infections, Sub-microscopic infections, Malaria, Ethiopia

Background

The Global Technical Strategy for Malaria of the World Health Organization (WHO) calls for a world free of malaria [1]. To contribute to this global vision and

encouraged by the substantial gains made in malaria control over the last two decades, Ethiopia has embarked on progressively eliminating malaria starting from low malaria transmission areas [2–6]. In the initial phase, 239 woredas (districts) were targeted for malaria elimination by the National Malaria Control and Elimination Programme of Ethiopia [5]. Preparations are underway to shift diagnosis and surveillance approaches from reducing malaria morbidity and mortality to detecting

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infections and measuring transmission in the selected woredas [2, 5].

Malaria elimination requires the detection and clearing of all *Plasmodium* infections. A relatively high prevalence of subpatent or low parasite density infections, that are often missed by conventional diagnostic methods (microscopy and rapid diagnostic tests) are being reported especially from low transmission settings [7–14]. Such asymptomatic, subpatent infections could be explained by acquired immunity in higher transmission settings [15]. Even in low transmission settings, asymptomatic and subpatent infections might play a role in transmission dynamics, hindering the progress of malaria elimination [14–18].

In Ethiopia, *Plasmodium falciparum* and *Plasmodium vivax* are the major reported malaria parasite species with rare reports of *Plasmodium malariae* and *Plasmodium ovale*. In 2017, out of a total confirmed malaria cases of 1,530,739, *P. falciparum* accounted for 1,059,847 cases and *P. vivax* for 470,892 cases [1, 2].

The current study assessed the magnitude of subpatent infections in northwestern Ethiopia, using samples collected in 2015 from a nationwide household survey of malaria endemic areas.

Methods

Study area

The study used Dried blood spot (DBS) samples collected as part of the National Ethiopian Malaria Indicator Survey conducted in 2015 (EMIS-2015) [20]. The survey was conducted during the peak of the malaria transmission season between September and December, 2015. Sample collection during EMIS-2015 was limited to areas below 2500 m elevation, with 85% of samples collected from areas below 2000 m elevation and the remaining 15% from areas between 2000 and 2500 m elevation. For the present study, samples were selected purposefully from the Amhara, Benishangul-Gumuz and Tigray Regions (Fig. 1). From the EMIS-2015 results, rapid diagnostic test (RDT) prevalence ranged from 10.4% in Benishangul-Gumuz, 1.9% in Tigray, to 1.1% in Amhara. A separate study [21] also found this geographical area to represent a range of malaria transmission from very low to moderate based on seroprevalence. Participants tested by malaria microscopy and RDT that provided DBS samples were selected from the EMIS 2015 samples repository at the Ethiopian Public Health Institute (EPHI) in Addis Ababa, Ethiopia.

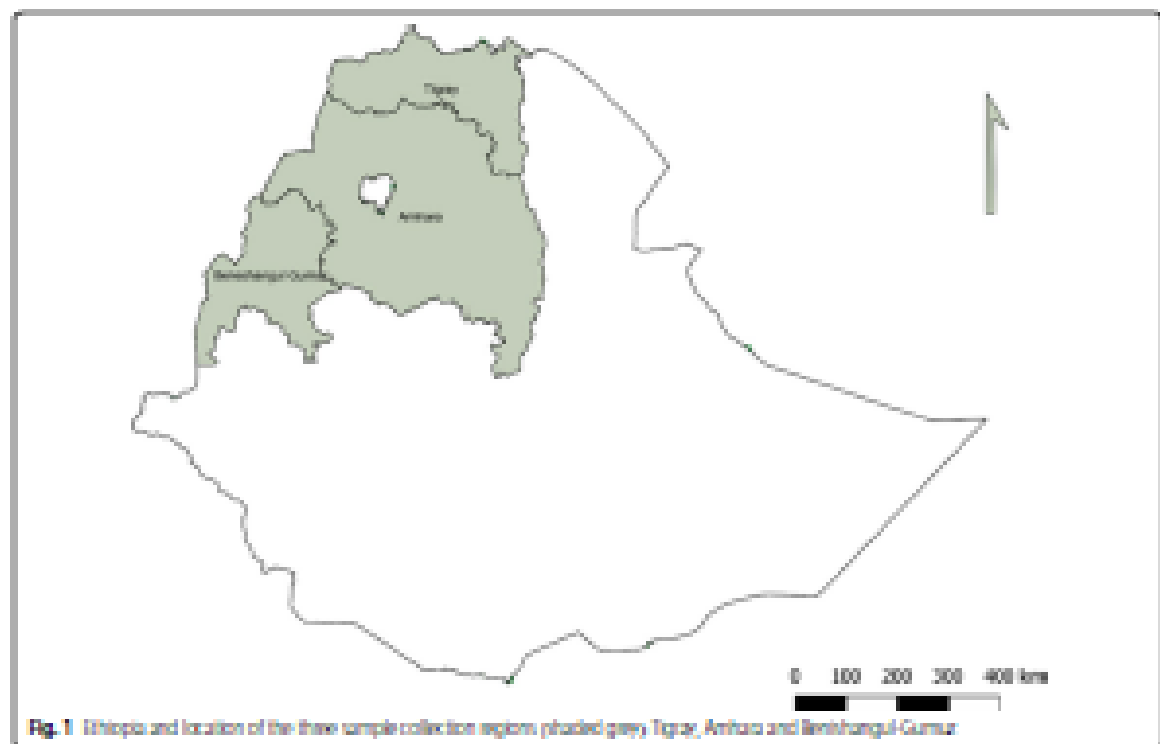


Fig. 1 Ethiopia and location of the three sample collection regions (shaded grey) Tigray, Amhara and Benishangul-Gumuz

Study population and sample collection

EMIS-2015 was a cross-sectional survey, which used a two-stage cluster sampling methodology [20]. Enumeration Areas (EA), the sampling unit, were selected proportional to population size and 25 households were selected by simple random sampling from each EA. Demographic, socioeconomic, malaria prevention, and malariometric data were also collected in the selected households. All children under 5 years of age in selected households and persons of all ages in every 4th household were eligible for biological sample collection and testing [20]. Whole blood from a single finger prick from consenting individuals (with or without fever) was collected for *Plasmodium* infection identification by RDT and microscopy, haemoglobin testing (Hemocue Hb 201+, Hemocue AB, Angelholm, Sweden) and for collection of DBS samples. Whatman 903 (GE Healthcare, Pittsburgh, PA) filter paper cards were used for DBS collection. These were air dried, individually packed in a plastic bag together with a desiccant and stored at -20°C at EPHI before they were sent to the U.S. Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia, for processing.

Plasmodium infection exposure

Serology methods and results for these samples have been previously reported [21]. Briefly, DBS elutions were assayed using bead-based multiplex assays for IgG antibodies for six.

***Plasmodium* antigens:** four human malaria species-specific merozoite surface protein-1 19 kD antigens (MSP-1)

and Apical membrane antigen-1 (AMA-1) for *P. falciparum* and *P. vivax*.

Parasite identification

Rapid diagnostic tests

The CareStart™ Malaria HRP2/pLDH (Pf/PAN) Combo RDT were used to detect malaria in the field. CareStart™ tests for histidine-rich protein 2 (HRP2) to detect *P. falciparum* and pan-*Plasmodium* lactate dehydrogenase (LDH) for *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*.

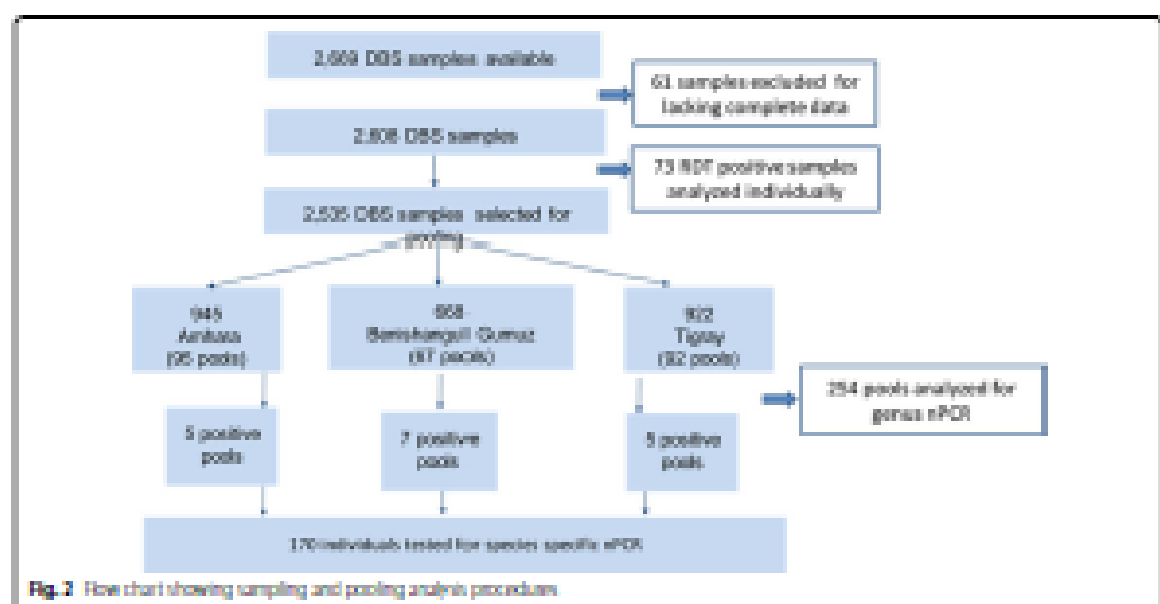
Microscopy

Thick and thin blood smears were made on the same slide, air-dried, and transported to EPHI. The slides were stained with 3% Giemsa for 10 min and screened for the presence of plasmodial infections. Microscopists were WHO certified and were blinded to the RDT and survey results. A slide was classified as negative if no *Plasmodium* asexual forms or gametocytes were found after viewing 100 fields. Any positive slide was confirmed by two other microscopists.

Polymersase chain reaction

The DBSs were shipped to CDC at ambient temperature. A total of 2535 RDT-negative and 2 samples without RDT results available from the three regions were pooled and analysed (Fig. 2). An additional 73 RDT-positive samples were analysed and confirmed by PCR individually.

Two-step pooling was used to estimate the prevalence of malaria in the RDT-negative samples, as previously



described by Zhou et al. [23]. A pilot was carried out to test for the effect of age (pooled by less than or equal to five and above 5 years of age) and different numbers of sample pooling (five or ten samples per pool), but no major differences by age or pooling size were found. Subsequently, individual samples from the same region were randomized into pools of ten samples.

DNA extraction A 6-mm disk was punched out from each DBS card and ten disks pooled for DNA extraction. Pooled DBSs were extracted with QIAamp DNA Mini kit as per the manufacturer's protocol (QIAGEN, Valencia, CA) with slight modification. Samples were left for an overnight incubation in ATL buffer plus Proteinase K to ensure proper lysis during pooling, and the DNA was then extracted following a normal extraction protocol. The purified DNA was tested using nested genus PCR (nPCR). For any pool with a positive PCR result, individual DBSs were extracted following the same protocol as for the pooled samples and species-specific PCR assays were performed.

nPCR detection for 18S ribosome RNA (rRNA) Purified DNA templates were used for amplification of the 18S rRNA gene subunit using a modified method as previously described [23]. Briefly, the 25 μ L PCR reaction contained 1 $\times$ PCR Master Mix (Promega, Madison, WI) and 100 nM of each PCR primer. For each primary PCR reaction, 5 μ L of DNA were used. The reactions were performed under the conditions of initial denaturation at 95 °C for 5 min, 25 cycles at 95 °C for 1 min, annealing at 58 °C for 2 min, and final extension at 72 °C for 2 min, followed by a final extension at 72 °C for 10 min. Species PCR was performed as per primary PCR except for use of 35 cycles and 2.5 μ L primary PCR product per reaction. Genus PCR was performed as per species PCR except annealing temperature was set at 64 °C. The amplified DNA products were stained with GelRed (Biotium, Hayward, CA), separated using 2% agarose gel electrophoresis and visualized under UV illumination.

Data analysis

Data were analysed using Stata 13 (Stata Corp. 2015, Stata Statistical Software: Release 14, College Station, TX: Stata Corp LP, USA). Descriptive statistics with 95% confidence interval were estimated for demographic characteristics. Multiple logistic regression was used to assess the association of risk factors (gender, age, elevation, bed net use and seropositivity) with nPCR parasitaemia results. Percent relative difference and concordance for microscopy and RDTs were calculated against PCR results as the reference standard. Values were considered significant at $P < 0.05$. A Venn diagram was constructed

using STATA to visualize the relationship amongst the four diagnostic methods: microscopy, RDTs, nPCR and serology. QGIS 2.18 was used to construct a map of the study area.

Results

Study population

Of the 2669 samples available from the three regions, 2535 samples were pooled for PCR analysis and 73 tested individually: 945 from Amhara, 668 from Benishangul-Gumuz and 922 from Tigray (Fig. 2). A total of 2608 samples had matched assay and survey data, comprising of 2535 RDT-negative, 73 RDT-positive and 2 without RDT results. About 53% (95% CI 50.4–54.2) of the samples tested were from females and 44% (95% CI 42.0–45.8) from children under 5 years of age (Table 1). The mean participant age from the samples was 16.1 years (95% CI 15.4–17.0) (range: 1–88 years). About 80% of the data were collected from areas below 2000 m in elevation. Of the 1354 samples with a paired seroprevalence result, almost half [51.3% (95% CI 48.6–53.9)] were seropositive of which 41.3% (95% CI 38.7–43.9) of the samples were seropositive for *P. falciparum* (MSP-1 or AMA-1 antigen responses) and 34.0% (95% CI 31.6–36.6) seropositive for

Table 1 Characteristics of survey samples from Tigray, Amhara and Benishangul-Gumuz Regions of Ethiopia, 2015

Characteristics	Number	Proportion (%) (95% CI)
Sex	N = 2608	
Female	1364	52.3 (50.4–54.2)
Male	1244	47.7 (45.8–49.6)
Age	N = 2608	
< 5	1145	43.9 (42.0–45.8)
5–14 years	528	19.9 (18.2–21.5)
15–24 years	335	12.8 (11.2–14.3)
25–49 years	460	17.6 (16.2–19.1)
> 50 years	130	5.0 (4.4–5.6)
Elevation	N = 2608	
< 2000 m	2104	80.7 (80.3–81.2)
$\geq$ 2000 m	494	18.7 (16.7–19.7)
Use of bed net	N = 1798	
Yes	1073	59.6 (57.3–61.9)
No	726	40.4 (38.1–42.7)
Seropositivity	N = 1354	
<i>P. falciparum</i> (MSP-1 or AMA-1)	559	41.3 (38.7–43.9)
<i>P. vivax</i> (MSP-1 or AMA-1)	461	34.0 (31.6–36.6)
<i>P. malariae</i> (MSP-1)	94	7.0 (5.7–8.4)
<i>P. ovale</i> (MSP-1)	66	4.9 (3.8–6.2)
Any Plasmodium (MSP-1 or AMA-1)	694	51.3 (48.6–53.9)

P. vivax (MSP-1 or AMA-1). Additionally, 7.0% (95% CI 5.7–8.4) and 4.9% (95% CI 3.8–6.2) of participants were seropositive for *P. malariae* and *P. ovale* MSP-1 antigen responses, respectively. Overall, 59.6% (95% CI 57.3–61.9) of respondents providing samples reported having slept under a bed net in the previous night (Table 1).

Prevalence of malaria infection

The detection of *Plasmodium* infection varied by diagnostic methods: microscopy, RDT and nPCR (Table 2). Of the 3608 samples tested 2.8% (95% CI 2.2–3.5) were found to be positive for *Plasmodium* using RDTs, comprising of 1.9% (95% CI 1.5–2.5) due to *P. falciparum* (only HRP2 positive), 0.4% (95% CI 0.2–0.7) due to *P. falciparum* infection with or without other *Plasmodium* species (HRP2 and panLDH positive) and 0.5% (95% CI 0.3–0.5) were *P. vivax*, *P. ovale* or *P. malariae* infection (only panLDH positive). Using microscopy, 1.2% (95% CI 0.8–1.7) of the samples were found to be positive for *Plasmodium*, of which 1.0% (95% CI 0.6–1.4) were due to *P. falciparum* infection, 0.2% (95% CI 0.0–0.4) were due to *P. vivax* infection and 0.08% (95% CI 0.0–0.3) were due to mixed *P. falciparum* and *P. vivax* infection. RDT positivity was over twofold higher than microscopy positivity (Table 2). Figure 3 shows the relationships and overlap among serology, RDTs, microscopy and nPCR methods.

Malaria DNA screening by nPCR detected 3.3% (95% CI 2.7–4.1) positivity for any malaria species. Of this, *P. falciparum* accounted for 3.1% (95% CI 2.5–3.8) of infection, *P. vivax* accounted for 0.4% (95% CI 0.2–0.7) of infection, mixed *P. falciparum* and *P. vivax* 0.1% (95% CI 0.0–0.4), and mixed *P. falciparum* and *P. malariae* 0.1% (95% CI 0.0–0.3). PCR detected 64.4% and 16.1% more

positives compared to microscopy and RDT, respectively ($P < 0.05$). In addition, PCR detected two *P. malariae* cases mixed with *P. falciparum* cases. By RDT, these were both appropriately positive for HRP2 and panLDH antigens. Using microscopy, one of the *P. malariae* positive infection was missed and the other was identified as mixed infection. No *P. ovale* infection was identified by any diagnostic method (Table 2). Seropositive samples had a very high likelihood of being screened positive for malaria DNA [adjusted OR 10.0 (95% CI 3.2–29.4; $P < 0.001$)]. While living in lower elevation areas (< 2000 m), sex, age group, or bed net use were not associated with PCR positivity in the study samples (Table 3).

PCR identified 30 additional samples as positive that were negative by RDT (Table 4). Of these, 25 were due to *P. falciparum* infection, five were due to *P. vivax* infection, and two were due to a mixed infection of *P. falciparum* and *P. malariae*. Half of the additional positive samples (14 of 30), and mixed *P. falciparum* and *P. malariae* cases were identified from samples collected in Benti-hangul-Gumuz, a region of higher malaria transmission in Ethiopia.

The percent concordance of RDTs and microscopy with nPCR detection on sample positivity varied between the three regions (Table 5). Overall, 80.8% (29/73) of the samples identified as positive by RDTs and 83.9% by microscopy were confirmed positive by nPCR. The highest agreement with nPCR data for both RDTs (90.7%) and microscopy (86.7%) was observed in the Benti-hangul-Gumuz samples where *P. falciparum* is predominant. In the Amhara and Tigray regions agreement with nPCR diagnosis was lower at 64.3% and 68.8%, respectively.

Table 2 Variation in malaria positivity of samples between RDT, microscopy and nPCR in the study area (Tigray, Amhara and Benti-hangul-Gumuz Regions), 2015

Diagnostic methods	Species diagnosed	Number of positives	Percent positivity (95% CI)	Percent relative change from PCR
RDT (n = 3608)	<i>P. falciparum</i> (HRP2 +)	50	1.8 (1.5–2.5)	–37.5
	<i>P. vivax</i> / <i>P. malariae</i> / <i>P. ovale</i> panLDH +)	12	0.5 (0.3–0.8)	0.0
	<i>P. falciparum</i> or mixed (HRP2 and panLDH +)	11	0.4 (0.2–0.7)	N/A
	Total <i>Plasmodium</i> positive	73	2.8 (2.2–3.5)	–16.1
	<i>P. falciparum</i>	26	1.0 (0.6–1.4)	–68.8
Microscopy (n = 3608)	<i>P. vivax</i>	4	0.2 (0.0–0.4)	–60.0
	Mixed <i>P. falciparum</i> + <i>P. vivax</i>	2	0.08 (0.0–0.3)	–31.3
	Total <i>Plasmodium</i> positive	31	1.2 (0.8–1.7)	–64.4
	<i>P. falciparum</i>	25	1.1 (0.7–1.6)	
	<i>P. vivax</i> mono-infection	10	0.4 (0.2–0.7)	
nPCR (n = 3608)	Mixed <i>P. falciparum</i> + <i>P. vivax</i>	3	0.1 (0.0–0.4)	
	Mixed <i>P. falciparum</i> + <i>P. malariae</i>	2	0.07 (0.0–0.3)	
	Total <i>Plasmodium</i> positive	37	3.3 (2.7–4.1)	
	<i>P. falciparum</i> mono-infection	25	3.1 (2.5–3.8)	
	<i>P. vivax</i> mono-infection	4	0.4 (0.2–0.7)	

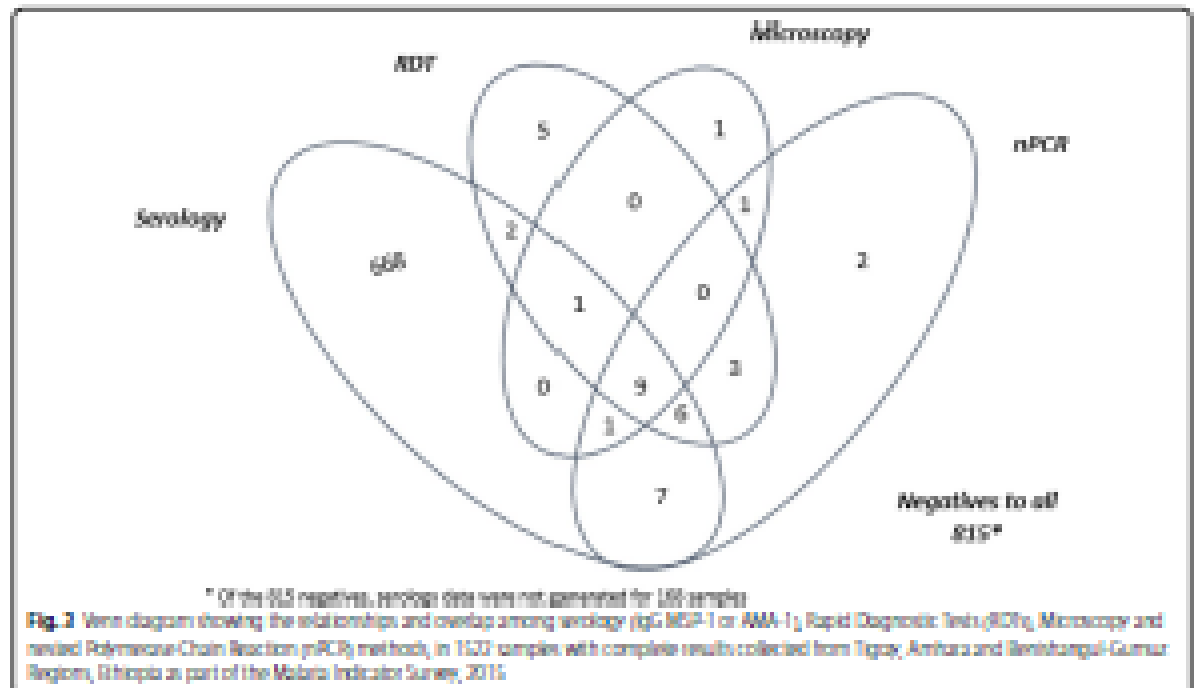


Table 2 Association between nPCR positivity and sample characteristics (sex, age, elevation, bed net use and seropositivity)

	PCR positive			
	Unadjusted OR (95% CI)	P-value	Adjusted OR (95% CI)	P-value
Sex				
Female	Ref		Ref	
Male	1.3 (0.8–2.0)	0.231	0.8 (0.5–1.2)	0.271
Age intervals (years)				
<5	Ref		Ref	
5–14	1.3 (0.5–3.8)	0.758	1.3 (0.5–3.4)	0.525
15–24	1.0 (0.3–3.3)	0.974	1.3 (0.4–3.6)	0.702
25–49	0.5 (0.2–1.8)	0.340	0.7 (0.2–3.8)	0.528
≥ 50	0.4 (0.1–1.2)	0.407	0.5 (0.1–3.8)	0.572
Elevation (m)				
> 2000	Ref		Ref	
< 2000	7.2 (1.0–53.0)	0.052	5.0 (0.1–53.2)	0.118
Use of bed net (N = 1758)				
No	Ref		Ref	
Yes	0.7 (0.4–1.3)	0.267	0.8 (0.4–1.3)	0.345
Seropositivity				
No	Ref		Ref	
Yes	5.8 (2.0–17.1)	0.001	10.0 (3.2–29.4)	< 0.001

Table 4 Malaria prevalence by nPCR from RDT-negative samples by region, 2015 EMIS

Region	RDT negative samples	PCR-positive samples				Total positive, % (n)
		<i>P. falciparum</i>	<i>P. vivax</i>	<i>P. malariae</i>	<i>P. ovale</i>	
Benishangul-Gumuz	668	12	2	2 ^a	0	2.1% (14)
Amhara	944	8	2	0	0	1.1% (10)
Tigray	621	5	1	0	0	0.7% (6)
Total	2533	25	5	2 ^a	0	1.2% (30)

71 samples that were RDT positive and 2 samples from Amhara and Tigray which not tested by RDT were excluded

^a Mixed with *P. falciparum*

Table 5 Concordance of RDTs and slide positive samples compared to nPCR by region, 2015 EMIS

Region	Percent concordant, % (n/N)	
	RDT versus PCR	Slide versus PCR
Benishangul-Gumuz	93.7% (29/31)	86.7% (13/15)
Amhara	64.2% (9/14)	77.8% (9/7)
Tigray	68.8% (11/16)	71.4% (7/7)
Total	83.8% (54/73)	83.8% (36/43)

Discussion

In this study, results of microscopy, RDT and nPCR detection methods were used to examine the prevalence of *Plasmodium* infection in malaria endemic settings in northwest Ethiopia. Nested PCR identified 30 additional positive malaria infections that were missed by RDTs in the field, and thus not provided treatment at the time of the survey. The prevalence reported by nPCR was 3.3%, 16.1% higher than that detected by RDTs and 64.4% higher than from microscopy. More than 60% of the nPCR positive cases were identified from Benishangul-Gumuz Region, which is one of the higher malaria transmission region in Ethiopia [5]. The region showed a similar high prevalence of malaria by microscopy and RDTs in this study and in a separate serology study of the same samples [21].

The study compared microscopy and RDT detection methods with a pooling nPCR approach that can detect parasite density as low as 0.1–10 parasites/μl of blood [22–26]. Close agreement was observed between both RDT and microscopy results compared to PCR with 80.8% and 83.9% concordance, respectively. Microscopy, considered the gold standard for malaria diagnosis [26], has a variable limit of detection (50–100 parasite/μl of blood) [26–28], depending on the skill of the technician and the reagents used [26–28]. As per the national malaria diagnosis and treatment guideline [29], Ethiopia uses microscopy in health centres and hospitals and RDTs in health posts (primary health

care units). In the current study with slide preparation and RDTs conducted under field conditions where test requirements may not be optimal, high variability was observed in the results across the three methods. This discordance becomes disproportionately higher in these low transmission settings with very few positive samples. The strong association between seropositivity and PCR positivity suggests a role for using multiplex seroprevalence results to more rapidly and inexpensively identify hotspots or confirm lack of *Plasmodium* infection in areas reporting low incidence.

Although the lower RDT positivity is likely due to low-density infections, the possibility of *hrp2/3* gene deleted *P. falciparum* infections has been raised in Ethiopia with alarming reports originating from Eritrea [39–43]. Although *HRP2* and *HRP3* deletions were reported recently from a study in Amhara [34], a study of larger geographic scope is currently ongoing in Ethiopia (Sindew Mekasha, EPHI, personal communication).

Several studies have reported asymptomatic *Plasmodium* infections in low transmission settings using PCR from a range of countries [7–15, 18, 22, 35, 36]. The terms asymptomatic, submicroscopic, and subpatent are often used interchangeably to describe a malaria infection that cannot be detected by conventional methods (microscopy and RDTs) but can be detected by more sensitive methods such as PCR. The prevalence of subpatent infection in low transmission setting has ranged from 0.003 to 44%, depending on the sensitivity of the tools used and the sample collection area [36]. Studies in Iran [37] and Sri Lanka [38] reported zero prevalence by the more sensitive methods, indicative of no local transmission and confirmation of malaria elimination. Gohara et al. [14] and Tadense et al. [15] reported PCR prevalence ranging from 1.7 to 5.8% in southwest Ethiopia, comparable to the 3.3% prevalence reported in the current study.

Subpatent infections, despite the low parasite density, could be infectious to mosquitoes [15, 19, 39].

Studies in The Gambia [40], Thailand [35], Peru [41] and Ethiopia [17] showed even low-density, asymptomatic infections could be infectious to mosquitoes, which may pose a potential, unidentified reservoir for malaria transmission. A recent review [19] summarized that lower density of parasites were seen in low transmission compared to high transmission settings and argued that subpatent infections contribute to the infectious reservoir, could be long-lasting, and predictive of future periods of patent infections.

Several new tools are being developed with higher limits of parasite detection in field settings, such as, ultrasensitive RDTs and loop-mediated isothermal amplification (LAMP) [35, 42]. The current study used a sample pooling methodology that decreases cost and time and is applicable to screening large number of samples, particularly from low transmission settings [23, 43]. The low blood volume eluted from the DBEs in the current study may limit parasite detection as the volume of blood analysed is critical in determining its limit of detection [44, 45]. Although more sensitive molecular methods have been developed [46], the cost and feasibility of the tests in field settings could limit their wide-scale adoption and uptake in resource-limited countries.

Conclusion

The current study reports the presence of subpatent infection in the samples collected as part of a national cross-sectional malaria survey in 2015. Using mPCR, the detection of malaria parasites increased by nearly threefold over rates compared to microscopy. Subpatent infections could potentially be infective to mosquitoes contributing to ongoing malaria transmission. Malaria elimination efforts may need to consider low-density, subpatent infections in managing sources of infectious reservoirs or hotspots, in addition to strengthening routine surveillance and response systems.

Abbreviations

AMA-1: Apical membrane antigen-1; CDC: Centers for Disease Control and Prevention; DBS: Dried blood spot; EA: Enumeration Area; EIMS: Ethiopian Malaria Indicator Survey; EPHI: Ethiopian Public Health Institute; HRP-2: Histidine-rich protein 2; MSP-1: Merozoite surface protein-1; mPCR: Nested polymerase chain reaction; pLDH: Plasmodium lactate dehydrogenase; RDTs: Rapid diagnostic tests; rRNA: Ribosome RNA; WHO: World Health Organization.

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Authors' contributions

Conceived the study: AA, AAA, WD, JH. Designed the experiments: AA, YPS, ZJ, BW. Performed the experiments: BW, IR, ZJ, SS, MS. Analysed the data: AA, BW, JH. Assisted study implementation: AA, AK, YPS, AAA, WD, HT, MWM, JH. Critically reviewed and contributed to writing: JH, LS, CW, YPS, BW, IR, ZJ,

AAA, MH, HT, MWM, CDWD. Drafted the manuscript: AA. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Ethical clearance was obtained from the Health Science College of Addis Ababa University (AAUM) on 2016. The GMS 2015 protocol received ethical clearance from the National Research Ethics Review Committee of Ethiopia. The current survey protocol which included molecular testing underwent human subjects review at CDC and was considered a non-research program evaluation activity.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Paper III. Glucose-6-phosphate dehydrogenase (G6PD) deficiency in Ethiopia: absence of common African and Mediterranean allelic variants in a nationwide study

Paper III

RESEARCH

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Glucose-6-phosphate dehydrogenase (G6PD) deficiency in Ethiopia: absence of common African and Mediterranean allelic variants in a nationwide study

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Abstract

Background: Building on the declining trend of malaria in Ethiopia, the Federal Ministry of Health aims to eliminate malaria by 2030. As *Plasmodium falciparum* and *Plasmodium vivax* are co-endemic in Ethiopia, the use of primaquine is indicated for both transmission interruption and radical cure, respectively. However, the limited knowledge of the local prevalence of glucose-6-phosphate dehydrogenase (G6PD) deficiency and its associated variants has hindered the use of primaquine.

Methods: Some 11,138 dried blood spot (DBS) samples were collected in 2011 as part of a national, household Malaria Indicator Survey, a multi-stage nationally representative survey of all malaria-endemic areas of Ethiopia. A randomly selected sub-set of 1414 DBS samples was successfully genotyped by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) technique. Considering the geographical position and ethnic mix of the country, three common variants: G6PD^A (A376G), G6PD^{A–} (G202A) and Mediterranean (C563T) were investigated.

Results: Of the 1998 randomly selected individuals, 1429 (71.5%) DBS samples were genotyped and merged to the database, of which 53.5% were from females. G6PD^A (A376G) was the only genotype detected. No sample was positive for either G6PD^{A–} (G202A) or Mediterranean (C563T) variants. The prevalence of G6PD^A (A376G) was 8.9% [95% confidence interval (CI) 6.7–11.2] ranging from 1.2.2% in the Southern Nations, Nationalities and Peoples' (95% CI 5.7–18.7) to none in Dire Dawa/Harari Region.

Conclusion: The common G6PD^{A–} (G202A) or Mediterranean (C563T) variants were not observed in this nationwide study. The observed G6PD^A (A376G) mutation has little or no clinical significance. These findings supported the adoption of primaquine for *P. falciparum* transmission interruption and radical cure of *P. vivax* in Ethiopia. As the presence of other clinically important, less common variants cannot be ruled out, the implementation of radical cure will be accompanied by active haematological and adverse events monitoring in Ethiopia.

Keywords: Ethiopia, Malaria, G6PD deficiency, Primaquine

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Background

In Ethiopia, malaria is caused by both *Plasmodium falciparum* (63.7% of all confirmed cases) and *Plasmodium vivax* (36.3%) [1]. Due to the scale-up of key malaria interventions in the last decade, several districts are now in the pre-elimination phase [2]. A drug capable of interrupting *P. falciparum* malaria transmission and radical cure of *P. vivax* has the potential to accelerate malaria control and elimination efforts. Primaquine, an 8-aminoquinoline, is currently the only generally available drug for these indications in malaria endemic countries. The widespread use of primaquine has been limited due to its potential to induce haemolytic anaemia in glucose-6-phosphate dehydrogenase (G6PD)-deficient individuals. Haemolytic anaemia in G6PD-deficient individuals can range from mild to life-threatening conditions [3–5]. Another 8-aminoquinoline, tafenoquine, completed Phase 2 and 3 trials and recently was approved by the US Food and Drug Agency for radical cure of *P. vivax* infections [6].

G6PD deficiency is an X-linked genetic disorder and it is estimated to affect more than 400 million people worldwide [4, 7]. It is one of the most prevalent polymorphisms and enzymopathies in humans and is found more commonly in malaria-endemic areas [4, 7]. In Ethiopia, there is limited information about the occurrence and distribution of G6PD deficiency [7]. There has been no nationally representative study on its prevalence and distribution conducted to date. Sporadic studies note the absence of G6PD deficiency in highland areas of the country, while in lowland areas G6PD deficiency prevalence ranged from 1.4 to 14.3% in the southwest (Nuer and Anuak) and northeast (Aregobas in Afar) regions concentrated in certain ethnic groups [8–11].

Because of substantial progress made in malaria control, Ethiopia's vision is to be malaria-free by 2030 [2, 12–14]. Determining the G6PD prevalence, distribution and variants can inform the Federal Ministry of Health's decision to adopt new policies to implement and scale-up the use of primaquine for both *P. falciparum* transmission reduction and *P. vivax* radical cure without prior G6PD testing, which will be crucial in achieving their vision. This study aimed to evaluate the genotypic prevalence of common G6PD deficiency allelic types in the general population residing in malaria-endemic areas of Ethiopia.

Methods

Study design and analysis

Dried blood spot (DBS) samples collected during the national Malaria Indicator Survey in 2011 (MIS 2011) were used for the current study. The MIS 2011 was a cross-sectional, multi-stage, representative household survey that produced national and sub-national estimates

for malaria-endemic and malaria prone areas of Ethiopia [15]. Using a two-stage cluster sampling methodology, 25 households were selected from each enumeration areas (average size 175 households) by simple random sampling. In each selected household, blood samples were collected for all children under 5 years of age and all family members in every fourth household [15].

A total of 11,138 DBS samples were collected and stored at -20°C as part of the MIS 2011 [15]. Representing the geographic regions that had been sampled during the survey, 1998 individuals were randomly selected. The MIS 2011 sampling protocol used a non-proportional allocation of samples proportional to population size. A sample size of 1998 was selected assuming a sample proportion of 0.03 to produce a two-sided 95% confidence interval with a width equal to 0.015 [16]. To ensure the representativeness of the samples, sampling weights were calculated by performing a weighting class adjustment to force weights from those tested for G6PD sub-sampling to equal the total weight in the full sample. Adjustments were made by province, altitude, and age group. Clustering at the village level was also accounted. PASS 11 (NCSS, LLC, Kaysville, Utah, USA) and SAS 9.4 (SAS Institute Inc, Cary, NC, USA) were used in sample size determination and data analysis, respectively.

Genotyping: PCR-RFLP

Based on a review of the literature, the country's geographic proximity to the Arabian peninsula and ethnic mix, the following three G6PD mutations common in Africa and the Mediterranean Regions were selected for genotyping: G6PD^A (A376G), G6PD^{A-} (G202A) and Mediterranean (C563T) [4, 7, 17].

A polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique was used for genotyping G6PD based on previous publications [19, 20] modifying the annealing temperature to reduce non-specific amplification. Briefly, DNA was extracted from DBS samples using the Qiagen DNA mini-kit (Qiagen, Germantown, USA). PCR was used for the amplification of three specific regions. Genomic DNA was first amplified using primers for G6PD^A (A376G), 5'-CCCAGGCCACCCCAGAGGAGA-3' (forward) and 5'-CGGCCCCGGACACGCTCATAG-3' (reverse) and all samples positive for G6PD^A (A376G) were then subjected to PCR amplification using primers for G6PD^{A-} (G202A), 5'-CACCCTGCCCCCTGTGACCT-3' (forward) and 5'-GGCCCTGACACCCACCCACCTT-3' (reverse). All DNA extracts were also amplified for Mediterranean (C563T) type mutation using primers 5'-AGCTCTGTGATCCTCACTCCCC-3' (forward) and 5'-GGCCAGGTGAGGCTCCTGAGTA-3' (reverse). The following PCR conditions were used for 376 and 202 mutations; initial

Table 1 Types of G6PD variants screened by RFLP and expected size of fragments in base pair

Mutation	Variants	Restriction enzymes	Size	Fragment Size		References
				Wild type	Mutant	
376 A→G	CoPD B→CoPD A	FokI	308	308	125, 183	[19]
202 G→A	A ⁺	NlaIII	211	211	83, 130	[19]
563 C→T	Mediterranean	MboII	285	33, 252	33, 98, 154	[18]

degeneration one cycle for 5 min at 94 °C, then 32 cycles for degeneration of 45 s at 94 °C, annealing of 30 s at 64 °C, extension of 45 s at 72 °C, and final extension at 72 °C for 1 min. Similar PCR conditions were used for Mediterranean (C563T) mutation with the following modifications; initial degeneration one cycle for 5 min at 94 °C, then 32 cycles for degeneration of 1 min at 94 °C, annealing of 1 min at 57 °C, extension of 1 min at 72 °C and final extension at 72 °C for 1 min.

Three restriction endonuclease enzymes, FokI (G6PD^A (A376G)), NlaIII (G6PD^A– (G202A)) and MboII (Mediterranean (C563T)) (New England Biolabs Ltd, Ipswich, MA), were used for the digestion of the amplified products (Table 1). Samples were incubated with the restriction enzymes for 1 h at 37 °C, followed by inactivation at 65 °C for 25 min [19–22]. Final PCR–RFLP products were separated using 2% agarose gel-electrophoresis and visualized under UV illumination (Fig. 1).

Known blood samples archived at CDC, collected from a cross-sectional survey conducted in Kenya and a malaria in pregnancy project conducted in Malawi served as positive controls for G6PD^A (A376G) and G6PD^A– (G202A) (African type mutations). However, there were no positive controls used for Mediterranean (C563T) type mutations. Results with faint and intermediate bands were repeated by using the concentrated DNA samples to achieve more distinct test results. The testing was conducted at the Malaria and other Parasitic Diseases Research Team at the Ethiopian Public Health Institute; a sub-set of samples (10%) was genotyped at CDC Atlanta for quality assurance testing and training.

Results

Of the 1998 randomly selected samples, only 1641 (82.1%) DBS were found. DNA was extracted and successfully amplified from 1548 samples (94.3%). An additional 119 samples were not able to be merged to the original database, resulting in 1429 merged genotype outcomes and 1414 with valid genotype results (15 samples with unsatisfactory gel results after repeated attempts). Of all the samples analysed and merged to the database, 53.5% (764/1429) were from females.

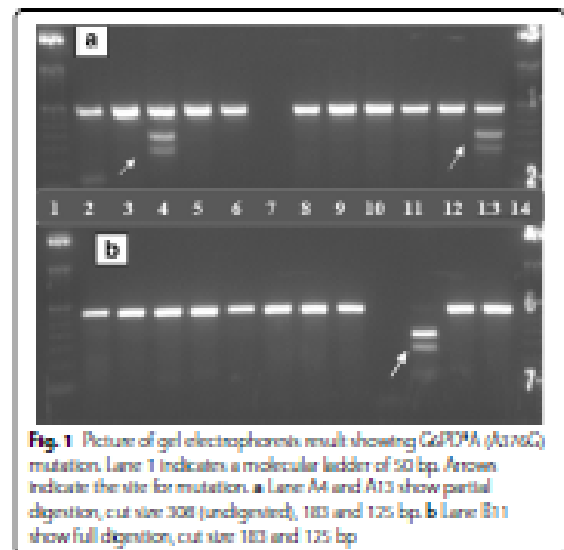


Fig. 1 Picture of gel electrophoresis result showing G6PD^A (A376G) mutation. Lane 1 indicates a molecular ladder of 50 bp. Arrows indicate the site for mutation. **a** Lane 4 and 13 show partial digestion, cut size 308 (undigested), 183 and 125 bp. **b** Lane 8 and 11 show full digestion, cut size 183 and 125 bp

Figure 2a shows the spatial distribution of the samples selected. Similar to the overall MIS 2011 estimates [15], the average age of the study participants was 14.3 (range 1–89) years. Although samples were randomly selected without considering signs and symptoms of malaria or malaria testing results, 19 samples were positive by malaria rapid diagnostic tests (15 *P. falciparum* and 4 *P. vivax*).

Table 2 shows the weighted genotyping results by region. The study showed that the G6PD^A (A376G) mutation was the only mutation found in 8.9% (95% confidence interval (CI) 6.7–11.2) of the analysed samples. Of the G6PD^A (A376G) mutations, 57.8% (75/130) was observed in females. The G6PD^A– (G202A) and Mediterranean (C563T) mutations were not detected. No spatial clustering was observed for the G6PD^A (A376G) mutation (Fig. 2b) which was similar to the spatial distribution of all samples selected (Fig. 2a).

Although not statistically significant, there was regional variation in the prevalence of the G6PD^A (A376G) mutation: the highest prevalence was observed in Southern Nations, Nationalities, and Peoples' and Tigray Regions (12.2%; 95% CI 5.7–18.7 and 12.0%;

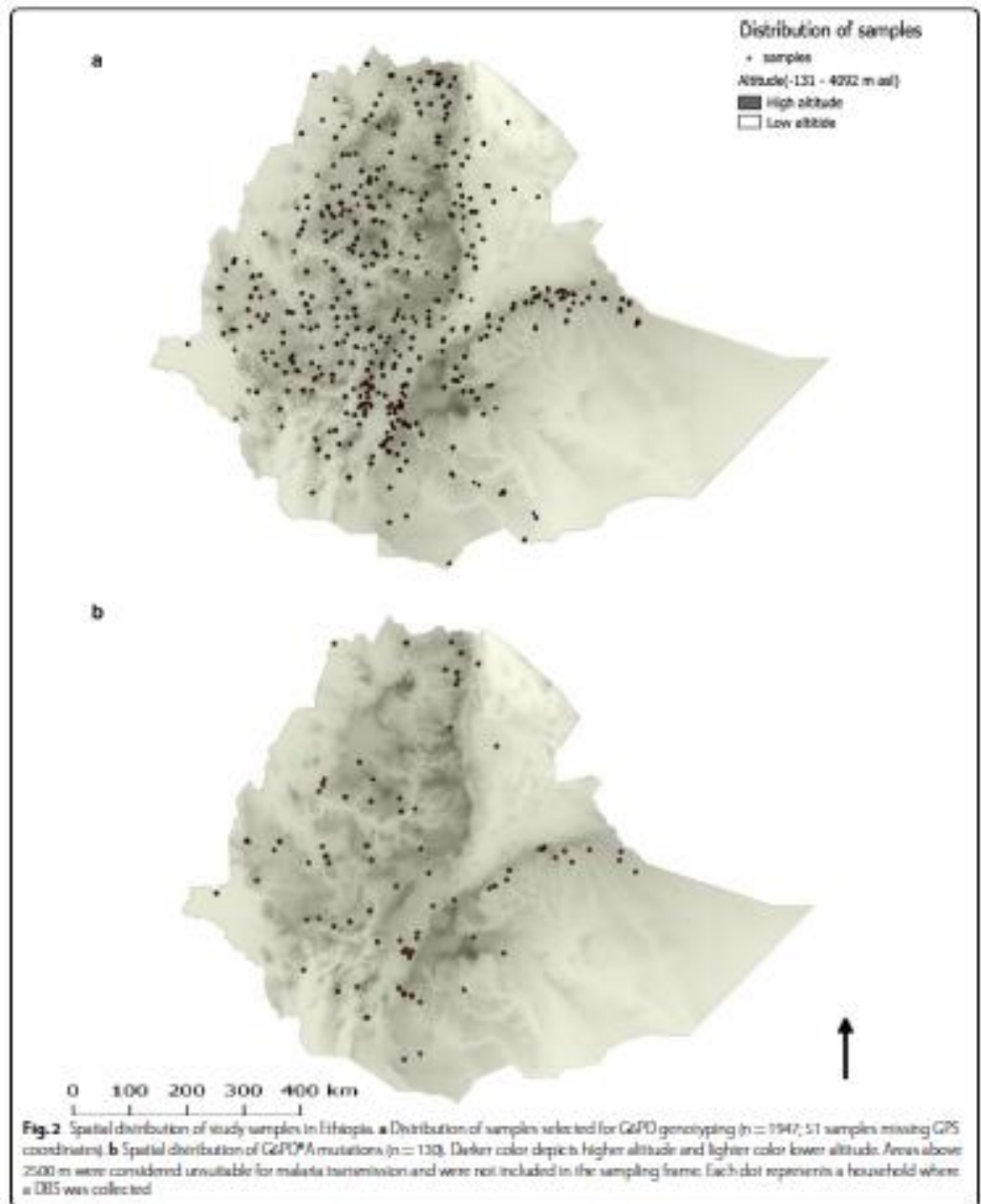


Table 2 Number of DBS samples analysed and prevalence of G6PD*A (A376G) mutants by region

Region	No. of samples extracted	No. of samples amplified	No. of samples merged for analysis	No. G6PD*A (A376G) mutants observed	Weighted prevalence (%) of G6PD*A (A376G) mutation (95% CI)
Afar and Somali	152	128 (84.2%)	118 (77.6%)	7	7.7 (2.5–14.9)
Amhara	302	262 (86.8%)	229 (75.8%)	15	7.5 (3.0–12.0)
Benchangul-Gumuz and Gambella	118	117 (99.2%)	109 (92.4%)	8	5.3 (2.7–10.0)
Dire Dawa and Harari	11	11 (100%)	10 (90.9%)	0	0
Oromia	640	633 (98.9%)	576 (90.9%)	58	8.0 (5.0–10.9)
Southern Nations, Nationalities, and Peoples'	277	256 (92.4%)	240 (86.6%)	28	12.2 (5.7–18.7)
Tigray	141	141 (100%)	132 (93.6%)	14	12.0 (4.4–19.5)
Total	1641	1548	1414	130	8.9 (6.7–11.2)

No G6PD*A– (G202A) and Mediterranean (C563T) variants were observed

95% CI 4.4–19.5, respectively), compared to Dire Dawa and Harari where no G6PD mutations were observed ($P=0.278$). The prevalence of G6PD*A (A376G) was 9.2% (95% CI 6.7–11.8) in areas with altitude less than 2000 m, and 7.9% (95% CI 2.4–13.4) in areas with altitude between 2000 and 2500 m above sea level ($P=0.403$).

Discussion

In this nationally representative study, 8.9% of the samples were noted to carry the G6PD*A (A376G) variant; the clinically relevant variants of G6PD*A– (G202A) and Mediterranean (C563T) were not observed. Although the more clinically important type G6PD*A– (G202A) is found in a sub-set of individuals with the G6PD*A (A376G) variant in other studies [5, 18, 23], none of the G6PD*A (A376G) mutant genes were noted to carry the G6PD*A– (G202A) mutation in this study. The G6PD*A (A376G) mutation observed in the study is a common variant, resulting in close to normal (~85%) enzyme activity of a non-deficient person, without significant clinical manifestations of G6PD-related haemolysis or appearing to confer resistance to malaria [5, 21, 24–27]. The analysis of Mediterranean (C563T) deficiency was included in this study because of the severity of the variant, the proximity of Ethiopia to the Mediterranean and Middle East Regions, and the ethnic mix observed in country [28, 29]; however, this severe variant was not observed.

The results are consistent with prior evidence of low G6PD deficiency prevalence in Ethiopia [7, 9–11, 25]. For example, the absence of G6PD*A– (G202A) and Mediterranean (C563T) mutations was recently reported from blood samples obtained from malaria patients in Southwestern Ethiopia, where 23% of the individuals were noted to carry the G6PD*A (A376G) mutation [22].

There are several limitations to this evaluation. First, the large sample loss (29.2%) mainly due to not being able to locate the corresponding DBS, is a major limitation, although there is likely little or no systematic bias introduced in this random loss. Second, the current study only tested for three common variants of the hundreds of known G6PD variants [25]. The current study cannot definitively conclude that the overall prevalence of clinically relevant G6PD-deficient variants is low or it excluded all clinically relevant variants found in Ethiopians (e.g., G6PD Rehovot [30] especially from regions with very few samples. Recent sequencing of samples from Southwestern Ethiopia have identified uncommon mutations, including a new mutation not previously reported [22], although their phenotypes were not documented [31]. Third, the current study only reports the genotypic prevalence of the variants which does not imply phenotypic prevalence. Tsegaye et al. [11] using the Carestart™ G6PD RDT reported high phenotypic prevalence of G6PD deficiency in Gambela Region (Anuak and Naer ethnic groups), thus, further studies are needed to identify the associated variants and haemolytic profile.

Conclusion

No common, G6PD deficiency variants (G6PD*A–) and Mediterranean (C563T) were found in this nationwide study. Following a review of the data from prior studies and the current study, the Federal Ministry of Health has adopted the addition of single low dose primaquine for transmission reduction of *P. falciparum*. In addition, they are adopting a policy for the first time in decades of primaquine use for radical cure of *P. vivax* infections without G6PD testing, starting in low transmission highland districts with directly observed therapy and close supervision [32]. Ethiopia, especially the highlands, may represent a lower risk location for the use of primaquine in

the radical cure for *P. vivax* malaria without G6PD testing. The safety of implementing this policy will be evaluated through active adverse events and haematological monitoring using a standardized approach with common endpoints [33]. The risks and benefits of primaquine radical cure without G6PD testing will need to be further assessed in Ethiopia as the *P. vivax* case management landscape evolves to incorporate developing regions in the lowlands, new evidence around higher dose and shorter course of primaquine and as new point-of-care, quantitative G6PD diagnostics and tafenoquine become available [6, 33–35].

Abbreviations

CI: confidence interval; DBS: dried blood spot; G6PD: glucose-6-phosphate dehydrogenase; MIS: malaria indicator survey; PCR: polymerase chain reaction; RFLP: restriction fragment length polymorphism; SNMPT: Southern Nations and Nationalities People's Region.

Authors' contributions

Conceived the study: AA, JH, RE. Designed the experiments: AA, YP, ZJ. Performed the experiments: AA, ZJ, WD, GA. Analyzed the data: AA, ZJ, YP, JH. Assisted study implementation: AA, WD, AK, GJ, TA, HT, HS, JM, JHL. Drafted the manuscript: AA. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Consent for publication

Not applicable.

Ethics approval and consent to participate

The MIS 2011 protocol received ethical clearance from the National Ethics Committee of Ethiopia as well as Emory University and RWTH institutional review boards. Additional ethical clearance for the G6PD study was obtained from the College of Health Science of Addis Ababa University (AAUMF-03-008). The survey protocol, which included G6PD testing, underwent human subjects review at CDC and received non-research determination (Human Subjects Research Tracking # 2015-3446).

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Annex 13. Assurance of Principal Investigator

This is an Original work no portion of the work referred to the dissertation has been submitted in support of an application for similar degree or qualification of this or any other University or other institute of learning.

Ashenafi Assefa (PI) -----

Supervisors:
